Conservation management of small populations of elephants (*Loxodonta africana*, Blumenbach 1797) in South Africa using genetics and population modelling.

by

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Declaration

I declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Date: Signature:

Thesis Abstract

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Many of the elephant populations in South Africa consist of small reintroduced populations in conservation areas smaller than 1000 km^2. In this dissertation I have analysed demographic and genetic factors which may affect extinction risk for these populations.

A modelling approach was used to determine the demographic factors that influenced the growth of the Pilanesberg National Park population. Demographic factors that could affect future population growth as well as harvesting, as a management action, were investigated. Previous and future growth rates were found to be higher than the long-term maximum of 7% per annum. Intercalving interval was the most sensitive demographic factor in future projections. Harvesting of adult males was found to be a very effective short-term management option for managing population growth. The stochastic models were found to be more representative of the real situation and are therefore more appropriate for further analyses of small populations.

Non-invasive DNA sampling techniques for population genetic studies of free-ranging animals have become increasingly popular, especially over the last 10 years. I conducted a pilot study to critically evaluate previously described faecal storage and faecal DNA extraction methods for efficiency and repeatability under local conditions. The quality and quantity of the faecal DNA obtained was evaluated by polymerase chain reaction (PCR) amplification of mitochondrial DNA (mtDNA) and microsatellite loci. Ideal dung storage and DNA extraction methods were identified. The problem of environmental contamination was highlighted during the amplification of the cytochrome *b* gene. Amplifications of the same samples three months after initial extractions were unsuccessful. Attempts to amplify DNA using elephant-specific mtDNA primers and microsatellites were unsuccessful. Under current conditions, I showed that obtaining sufficient quantities and quality of DNA from elephant dung was not possible.

Small populations are more likely to suffer from genetic problems, such as the loss of genetic diversity and inbreeding. I assessed the microsatellite diversity in four reintroduced elephant populations and compared them to the populations in the Kruger National Park (one of the source populations) and the Addo Elephant National Park (known to have undergone a severe bottleneck). Microsatellite genetic diversity of the reintroduced populations showed similar levels of heterozygosity and greater allelic richness than that of the Kruger population. MtDNA control region diversity within the southern African region was investigated, and was within the range observed in other African elephant populations. MtDNA haplotypes were shared between southern and eastern Africa, but, the two distinct haplogroups found in previous studies could not be identified in our data set. The failure to consider genetic problems in population management was highlighted by the reduced genetic diversity of the Addo population. I provide a list of genetic considerations which should be kept in mind when managing these populations.

For Franky

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"When eating an elephant, take one bite at a time"

Creighton Abrams

(1914 – 1974)

Chapter 1

General introduction

Problems faced by small populations

Although deterministic factors such as over-harvesting and habitat destruction lead to the reduction of previously large populations (Lacy 2000a), it is not necessarily these factors which lead to population extinction (Lacy 2000a; Miller & Lacy 2003). Stochasticity has a large impact on the viability of small populations. The reason for this stems from the fact that chance events become less predictable as the number of individuals in a population decreases (Lacy 2000a; Miller & Lacy 2003, Lande *et al*. 2003). The variance around the mean increases as the sample size (i.e. number of individuals) decreases. As a result large fluctuations in births, deaths, sex ratio and genetic variation will be experienced by small populations which may increase the population's extinction risk (Lacy 2000a; Miller & Lacy 2003; Lande *et al*. 2003).

Several stochastic factors have been identified as potentially affecting the viability of small populations. These include demographic stochasticity, environmental stochasticity, genetic stochasticity, and natural catastrophes (Shaffer 1981; Lande 1993; Caughley 1994; Lacy 2000a; Lande *et al*. 2003; Melbourne & Hastings 2008). Demographic stochasticity refers to the random variation in the number of births and deaths as well as variation in sex ratios (Caughley 1994). Environmental stochasticity is the result of temporal fluctuations in weather conditions as well as other factors that affect a population's environment such as variations in competitors, predators, parasites, and disease (Shaffer 1981; Lacy 2000a; Festa-Bianchet *et al*. 2006). Genetic problems in small populations may be due to a decrease in fitness because of inbreeding as well as the loss of genetic diversity due to random genetic drift, population bottlenecks, or founder effect (Hartl 1988; Lacy 1997; Meffe & Carroll 1997; Delport *et al*. 2001; Frankham *et al.* 2002; Frankham 2010a, b). Natural catastrophes, although often considered part of environmental stochasticity (Caughley 1994; Lacy 2000a; Lande *et al*. 2003), involve extremes of environmental variation which occur randomly such as floods, fires, droughts, and disease epidemics (Shaffer 1981; Coltman *et al*. 1999; Lacy 2000a; Rijks *et al*. 2008; Woolley *et al*. 2008a, b). All these factors are not necessarily mutually exclusive, and may at times influence each other or even compound the effect of a single factor (Lacy 2000a).

Which of these stochastic factors is likely to have the greatest effect on a population's viability is difficult to determine, and has led to much debate in conservation circles (Lande 1988; Lande 1993;

Caughley 1994; Lande 1995; Hedrick *et al*. 1996; Vucetich & Waite 1999; Clarke & Young 2000; Lacy 2000a; Brook *et al*. 2002b; Reed *et al*. 2002; Frankham 2003; Spielmann *et al*. 2004; Frankham 2005; O'Grady *et al*. 2006). Fortunately, as conservation biology is maturing, scientists are realising that these factors should not be analysed in isolation (Soulé 1985; Clarke & Young 2000; DeSalle & Amato 2004). A combined approach to interpreting these factors is necessary for the future management of populations, as for example the small populations of reintroduced African elephant, *Loxodonta africana* (Blumenbach 1797), in South Africa.

History of reintroduced elephant populations

Ivory hunting during the late eighteenth and nineteenth centuries led to the virtual disappearance of elephants from southern Africa (Douglas-Hamilton 1987; Skinner & Chimimba 2005). In South Africa elephant numbers continued to decrease reaching about 120 animals in 1920 (Hall-Martin 1992). Remnant populations were saved from extinction through the proclamation of game reserves (Hall-Martin 1992; Mills & Hes 1997). These remnant populations were found at Addo Elephant National Park (AENP), Kruger National Park (KNP), Knysna area of the Western Cape, and the Tembe area of KwaZulu-Natal (Hall-Martin 1992). Due to the protection of these populations, elephant numbers increased steadily and reached such numbers that certain populations (namely KNP) had to be culled to maintain carrying capacity in parks and to prevent the transformation of habitats (Bosman & Hall-Martin 1986; Mills & Hes 1997; Whyte 2001).

From the late 1970s, juveniles (2-12 years) from culling operations in KNP were sold in groups of similarly aged conspecifics and translocated to newly established conservation areas (Ebedes *et al.* 1991; Garaï 1997; Whyte 2001). Many of these conservation areas represent small areas of land, generally less than 1000 km^2, which were reclaimed from areas that were previously used for agriculture (Hall-Martin 1992). These areas were repopulated with animals that historically occurred there. In this way well over 800 elephants have been introduced into 58 conservation areas since 1979, including the populations in the current study, namely Pilanesberg National Park, Hluhluwe-iMfolozi Park, Madikwe Game Reserve and Phinda Game Reserve (Garaï *et al*. 2004). See Figure 1 for the distribution of elephants in South Africa and the location of the study populations. In 2003 the largest of these reintroduced populations numbered 387 individuals in a fenced area of approximately 900 km^2 (Hluhluwe-iMfolozi Park, Mackey *et al*. 2006) and one of the smallest populations numbered 10 individuals in a fenced area of approximately 80 km^2 (Mabula Game Reserve, Mackey *et al*. 2006). These population sizes are in stark contrast to more 'natural' elephant populations, which number in the thousands (Slotow *et al*. 2005; Mackey *et al*. 2006).

The founding populations for many of these small reserves comprised small numbers of juveniles with female-skewed sex ratios (Whyte 2001; Garaï *et al*. 2004; Slotow *et al.* 2005; Mackey *et al*. 2006). These populations currently consist of young adults, resulting in the problem of high growth rates and almost zero mortalities due to effective protection (Slotow *et al*. 2005; Mackey *et al*. 2006). The female-skewed sex ratio moreover results in a high growth potential (Slotow *et al*. 2005; Mackey *et al*. 2006). As a result of skewed sex ratios and the large number of young individuals, many of these populations have also suffered from deviant behaviours (Garaï 1997; Slotow *et al*. 2000; Slotow & van Dyk 2001; Slotow *et al*. 2001; Pretorius 2004). For example, adolescent bulls in Pilanesberg National Park killed more than 40 white rhinoceroses (*Ceratotherium simum*, Slotow *et al*. 2000; Slotow & van Dyk 2001). Furthermore, stochastic factors will affect the viability of these small populations (Shaffer 1981; Lande 1993; Caughley 1994; Lacy 2000a). Another compounding problem is that of founder effect which may result in the loss of genetic diversity due to the small number of founding individuals (Meffe & Carroll 1997; Delport *et al*. 2001).

Pilanesberg National Park

Pilanesberg National Park (PNP; 25°12'S, 27°06'E) is situated in the remains of an extinct volcano in the North-West Province of South Africa (Fig. 1). The park comprises 570 km^2 of fenced-in hilly savanna terrain. The habitat consists of *Acacia* and broad-leaf bushveld which ranges from thickets to open grassland patches. Water is readily available in the park due to a major river system (Mankwe River) that runs in a south-easterly direction through the central part of the park, as well as a large dam in the centre of the park and several smaller dams scattered throughout the park. Annual rainfall is approximately 630 mm, and falls mainly in summer. Winters are cold (minimum temperature 1°- 5°C) and summers are very hot (maximum temperature 28°- 31°C) (Slotow & van Dyk 2001; van Dyk & Slotow 2003).

Since the proclamation of the park in 1979, numerous animal species, including elephant, were reintroduced into PNP. Five elephants were initially introduced from AENP in 1979, but three individuals died shortly after the translocation, one was returned to AENP, leaving only one young male in PNP (Anderson 1994). A further 18 infants were introduced in 1981 from KNP (Anderson 1994). In 1982, two tame, circus-trained adult females (originally from KNP) were introduced and they assumed the role of matriarchs (Bosman & Hall-Martin 1986). Further introductions from KNP included 26 and 36 individuals in 1983 and 1993 respectively, and the first calf was born in 1989 (Anderson 1994; Slotow & van Dyk 2001).

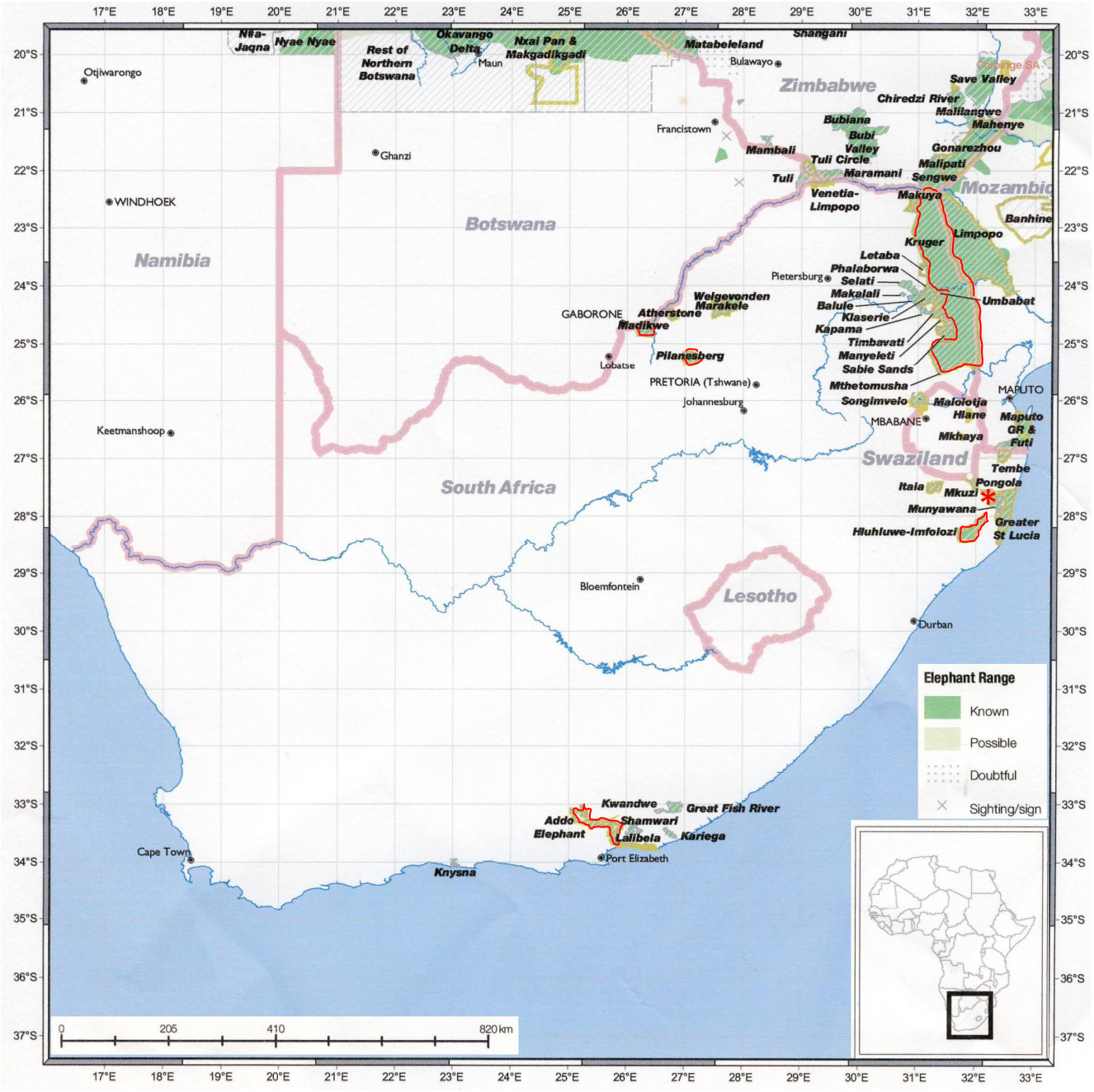

Figure 1 Distribution of elephants in South Africa as determined in 2006 (Blanc *et al*. 2007). The study populations of Kruger National Park, Addo Elephant National Park, Pilanesberg National Park, Hluhluwe-iMfolozi Game Reserve and Madikwe Game Reserve are highlighted in red, Phinda Private Game Reserve is indicated by a red *. Map modified from the *African Elephant Status Report 2007* (Blanc *et al*. 2007).

In the early 1990's young elephant males were found to be a major cause of mortality of white rhino (Slotow & van Dyk 2001). Males were found to be entering musth (a state of heightened sexual and aggressive activity in male elephants, Poole & Moss 1981) at a much earlier age than is found in natural populations. In 1998 six adult bulls were introduced into the park to stabilise the bull hierarchy in PNP (Slotow *et al*. 2000; Slotow & van Dyk 2001). In 2005 an uncontrolled wildfire spread through the reserve resulting in 29 deaths (ranging from infants to adults) and 18 injured elephants (Woolley *et al*. 2008b). The population, post-fire 2005, totalled 136 individuals of which 34 were independent bulls and 102 were breeding herd individuals in 18 family groups (Woolley *et al.* 2008b).

Hluhluwe-iMfolozi Park

Hluhluwe-iMfolozi Park (HiP; 28°11'S, 32°01'E) is situated in the north-eastern region of KwaZulu-Natal Province, South Africa (Fig. 1). The park comprises three areas: Hluhluwe Game Reserve, iMfolozi Game Reserve and the conservation corridor between the two reserves, totalling a fenced-in area of 890 km^2 (Boundja & Midgley 2009). The area is hilly with the main rivers being the Hluhluwe, Black and White Umfolozi. The habitat is highly heterogeneous, ranging from open grasslands to closed *Acacia* and broad-leaf woodlands (Whateley & Porter 1983; Archibald *et al.* 2005). There is an annual rainfall gradient across the reserve from 635 mm in the south-west to 990 mm in the north-east, and mean minimum and maximum temperatures are 13°C and 35°C, respectively (Whateley & Porter 1983).

The first reintroduction took place in 1981 with eight elephants from the KNP, although four died the same year (Dominy *et al*. 1998). By 1994, 184 elephant had been reintroduced, of which most were young animals from culling operations in the KNP. The first calf was born in 1990 (Dominy *et al*. 1998). Young male elephants were found to be involved in the killing of white and black rhino (*Diceros bicornis*) between 1991 and 2001 (Slotow *et al*. 2001). Following on from the success of introducing adult bulls in PNP (Slotow *et al*. 2000), 10 adult bulls from KNP were introduced in 2000 (Slotow *et al*. 2001). Elephants were translocated from Hluhluwe-iMfolozi to the eastern shores of St. Lucia Wetland Park in 2002 (R. Slotow, Amarula Elephant Research Programme, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication). In 2003 the HiP population was estimated to have 387 individuals (Mackey *et al*. 2006).

Madikwe Game Reserve

Madikwe Game Reserve (24°43'S, 26°10'E) is situated against the Botswana border in the North-West Province, South Africa (Fig. 1). The park comprises 620 km^2 of fenced-in mixed Kalahari

thornveld, shrub bushveld and mixed bushveld (Acocks 1988; Trinkel *et al*. in press), with the perennial Marico River bordering the reserve on the East and the Dwarsberg mountains bordering the South (Mosidi 1996). Mean annual rainfall ranges from 475 to 520 mm (Trinkel *et al*. in press), with mean minimum and maximum temperatures of 21°C and 35°C, respectively.

The park was proclaimed in 1991, and from 1992 well over 8000 animals, including elephant, were reintroduced into the reserve. The first reintroduction in 1992 involved the translocation of 31 sub-adult elephants from the KNP (R. Slotow, Amarula Elephant Research Programme, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication; Mackey *et al*. 2006), followed by 194 elephants, most as family groups, from Gonorezhou National Park, Zimbabwe in 1993 (Hofmeyr 2001; Mackey *et al*. 2006). Eight adult bulls were reintroduced from KNP between 1998 and 1999 (Hofmeyr 2001; Mackey *et al*. 2006). Six of the original KNP elephants were culled or hunted between 2000 and 2001 (R. Slotow, Amarula Elephant Research Programme, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication). Due to population growth, 16 elephants were translocated from Madikwe to Angola in 2000 (van Hoven & du Toit 2001), and 29 elephants were translocated to other game reserves in South Africa in 2001 (Grobler *et al*. 2008). In 2005 the population was estimated at 455 individuals (Blanc *et al*. 2007).

Phinda Private Game Reserve

Phinda Private Game Reserve (27º44'S, 32º25'E) is situated in the northern region of KwaZulu-Natal Province, South Africa (Fig. 1). The reserve comprises 150 km^2 of sweet lowveld bushveld, natal lowveld bushveld, coastal bushveld-grassland and sand forest (Druce *et al*. 2006). One perennial river (Mzinene River) flows from west to east through the southern section of the reserve and six dams are distributed throughout the reserve. Phinda has a summer rainfall regime of 750 mm per year (Shannon *et al.* 2009) and temperatures range from a minimum of 10°C to a maximum of 35°C (Druce *et al*. 2006). In 2004 the Munyawana Conservancy was formed by removing the fences between Phinda and the two neighbouring reserves, Zuka and Mziki Pumalanga (Druce *et al*. 2008).

Initial elephant reintroductions took place from 1992 to 1994, totalling 54 sub-adult elephants (less than 10 years old) from culling operations in KNP. Two bulls and two cows (approximately 20 to 25 years old) were introduced from Gonorezhou National Park, Zimbabwe in 1994. In order to normalise the population structure (Slotow *et al*. 2000; Druce *et al*. 2006), three adult bulls from Sabi-Sands, South Africa, were introduced in 2003 (Druce *et al*. 2006). A total of 37 elephants from four family groups were translocated from Phinda to other reserves in South Africa in 2003 (Druce *et al*. 2006). In

2005 the population totalled 81 individuals, including 19 independent bulls and five family groups (Druce *et al*. 2008). Since these reintroduced populations are not necessarily behaving like natural wild populations, a modelling approach can be used to analyse aspects of population growth as well as investigate management questions (Starfield & Bleloch 1991; Owen-Smith *et al*. 2006).

Modelling elephant population growth

Studies into population growth rates of reintroduced elephant populations have shown that these populations are growing at rates ranging from 4% to 16%, with more than half of the populations having growth rates above 7% (Slotow *et al*. 2005; Mackey *et al.* 2006). Calef (1988) calculated the long-term maximum growth rate of elephant populations to be 7%, with the following assumptions: a 50 : 50 sex ratio among all individuals in the population, an initial age structure of 50% adults : 50% juveniles, all females in the adult age class as reproductively active, 11 years as the female's age at first calving and three years as the mean calving interval. Many of the reintroduced populations do not follow these assumptions. The founding populations for many small reserves consisted of small numbers of juveniles with female-skewed sex ratios (Whyte 2001; Garaï *et al*. 2004; Slotow *et al*. 2005; Mackey *et al*. 2006), and now consist of young adults resulting in the problem of high growth rates and almost zero mortalities (Slotow *et al*. 2005, Mackey *et al*. 2006). Groups of similarly aged individuals will reach maturity at the same time, resulting in 'waves of births' causing an increase in population growth (Dominy *et al*. 1998; Slotow *et al*. 2005; Mackey *et al*. 2006).

Due to the unexpected growth rates of these small populations there is a need to understand the demographic factors that affected population growth (Slotow *et al*. 2005; Mackey *et al*. 2006). In this context, modelling is an ideal tool for analysing various aspects of a population (Starfield & Bleloch 1991). Similarly, several management questions can be simulated and their likely consequences determined (Owen-Smith *et al*. 2006). Furthermore, future projections of these populations can be used to investigate conservation concerns and can aid in making well-informed management decisions. Various modelling methods have been developed for the analysis of single-species populations; these include deterministic, stochastic, age-structured, and individual-based models (Starfield & Bleloch 1991; Uchmański & Grimm 1996). My analyses involved the use of a deterministic age-structured model, a stochastic individual-based model, and a stochastic population model using individual-based Monte Carlo simulations.

The Leslie matrix model is one of the simplest methods to model population dynamics (Leslie 1945). This is a deterministic age-structured model where the current female age structure and age-specific

survival and fertility rates are used to determine the future age structure of female animals (Jeffers 1982, Caswell 2001, Gallego 2003). The simplicity of the matrix algebra used has allowed this model, and subsequent modifications, to be used in a wide variety of situations in ecology including predator-prey relationships, patch dynamics, population interactions, and density dependence as well as in a variety of species ranging from plants to insects to long-lived mammals (Jeffers 1982; van Grenendael *et al*. 1988; Caswell 2001). Advantages of using the basic Leslie model include its requirement for very little data and its mathematical simplicity (Jeffers 1982; Allendorf & Ryman 2002), however the model is deterministic, density feedback is ignored, and individual variability within each age-class is not considered (Wu & Botkin 1980; Jeffers 1982; Uchmański & Grimm 1996; Caswell 2001; Allendorf & Ryman 2002; Gallego 2003).

Stochasticity is considered more appropriate for modelling long-lived species that are subject to environmental and demographic variability (Wu & Botkin 1980; Engen *et al*. 1998; Sukumar 2003). Wu and Botkin (1980) presented the first stochastic model designed specifically for studying the dynamics of African elephants. This model is a discrete-time stochastic model where a female's biological state at each age is considered; namely immaturity, maturity, pregnancy or anoestrus lactation (Wu & Botkin 1980; Gallego 2003). Stochasticity is brought in by considering the probabilities of conception and survival, which are functions of age, biological, and environmental factors (Wu & Botkin 1980; Mackey *et al.* 2006). Compared to the Leslie model, this model is much more realistic; however disadvantages include the need for more detailed data, especially for females (Wu & Botkin 1980; Gallego 2003; Sukumar 2003). This model has also been used to investigate the future growth of small elephant populations (Mackey *et al*. 2006; Woolley *et al*. 2008b), and the effect of age-specific mortality on elephant populations (Woolley *et al*. 2008a).

VORTEX is a stochastic, population modelling programme using individual-based Monte Carlo simulations. Population dynamics are modelled as discrete, sequential events that occur according to defined probabilities (Lacy 1993). This model includes demographic stochasticity (birth, sex, reproduction, death), environmental stochasticity (by modelling annual fluctuations in birth and death rates using user-defined probabilities), catastrophes (with specified probabilities causing one-year reductions in reproduction and survival), as well as genetic stochasticity (by reducing the survivorship of inbred individuals) (Lacy 1993; Lacy 2000b). This model is an improvement on the Leslie and Wu & Botkin models in that genetic stochasticity is included.

A further advantage is that VORTEX is a well-known generic population viability analysis (PVA) model, which has undergone extensive testing (Bustmante 1996; Lacy 2000a). Increasing reserve

sizes, immunocontraception, distortion of sex ratios in order to decrease growth potential, translocation, culling, and hunting have been suggested for the management of elephant populations (Slotow *et al*. 2005; Delsink *et al.* 2006; Owen-Smith *et al*. 2006). VORTEX can be used to simulate these management actions; the harvesting option can be used to mimic hunting, culling, and the removal of animals for translocation, with supplementation simulating translocations or releases from captive breeding programmes (Miller & Lacy 2003).

The use of generic PVA models has however received some criticism (Lacy 2000a). The expansion of desktop computers has made the use of generic models more popular, particularly for biologists who are not skilled computer programmers; however this can lead to problems when the user does not understand the basic structure of the model, thus making the computer adage of "garbage in, garbage out" a reality (Lacy 2000b; Beissinger 2002; Miller & Lacy 2003; Patterson & Murray 2008). Stochastic individual based models require a lot of information regarding species biology, however the data required from many endangered species is insufficient or absent, leading to unreliable predictions (Beissinger & Westphal 1998; Coulson *et al*. 2001; Beissinger 2002; Ellner *et al*. 2002; Doak *et al*. 2005; Bakker *et al*. 2009). Associated with the lack of sufficient data is the assumption that future growth rates will be similar to those observed; making catastrophes and processes that change key vital rates difficult to parameterise (Coulson *et al*. 2001). Furthermore the predictive accuracy of models is difficult to determine (Coulson *et al*. 2001; McCarthy *et al*. 2001); various studies have compared a range of generic PVA packages in order to determine whether standardized models using these packages can produce congruent predictions (Lindenmayer *et al*. 1995; Mills *et al*. 1996; Armbruster *et al.* 1999; Brook *et al*. 1999; Brook *et al*. 2000a, b; Chapman *et al*. 2001; Strem 2008), however the accuracy of predictions, using retrospective analyses, has only been assessed in a handful of studies (Brook *et al*. 1997; Brook *et al*. 2000b; Ball *et al*. 2003). Despite the many uncertainties, PVA models are useful as a conservation management tool even when information is known to be lacking (Brook *et al*. 2000b; Beissinger 2002; Brook *et al*. 2002a; Reed *et al*. 2002; Norris 2004; Ben-Ami *et al*. 2006; Zhang & Zheng 2007; Leimgruber *et al*. 2008; Jarić *et al*. 2009). Another factor that needs to be considered in small populations is that of genetic diversity.

Conservation genetics

Conservation genetics deals with "the genetic factors that affect extinction risk and genetic management regimes required to minimise these risks" (Frankham *et al*. 2002). Small populations are more likely to suffer from genetic stochasticity, which can increase extinction risk by affecting fitness and evolutionary potential (Meffe & Carroll 1997; Frankham 2005; Bouzat 2010; Frankham 2010a).

Reintroduced elephant populations are small populations which can be affected by founder effect, population bottlenecks, and a lack of exchange of genetic material between populations which can result in genetic drift and affect genetic diversity (Frankham *et al.* 2002; DeSalle & Amato 2004; Frankham 2010b). Small populations can suffer from founder effects since the founding individuals may not comprise the entire genetic diversity of the original population (Mayr 1963); tusklessness in the majority (97%) of cows in AENP has been proposed to be due to founder effect (Whitehouse 2001). Populations maintained at a small number for several generations (population bottleneck) can suffer a further loss of genetic diversity, particularly the loss of rare alleles (Nei *et al*. 1975: Cornuet & Luikart 1996), as seen in other African mammals which have undergone population bottlenecks, for example, roan antelope (*Hippotragus equines*, Alpers *et al*. 2004), black wildebeest (*Connochaetes gnou*, Alais 2000; Grobler *et al*. 2005) Cape mountain zebra (*Equus zebra zebra*, Moodley & Harley 2005), kob antelope (*Kobus kob*, Lorenzen *et al*. 2007), southern white rhino (*Ceratotherium simum simum*, Scott 2008) and walia ibex (*Capra walie*, Gebremedhin *et al*. 2009). Genetic drift can lead to a loss of genetic diversity, random changes in allele frequencies and differences between populations (Frankham *et al*. 2002), as seen in the AENP elephant population (Whitehouse & Harley 2001) and in Cape mountain zebra (Moodley & Harley 2005).

These populations may also, by chance, accumulate deleterious alleles which can increase in frequency particularly if mating occurs between closely related individuals (inbreeding). Inbreeding depression, the deleterious effects of inbreeding, results in a reduction in reproduction and survival (Ralls *et al*. 1988). Despite inbreeding depression not currently observed in elephant (Whitehouse 2001), it has been documented in an introduced population of lion in HiP (Trinkel *et al*. 2008), and other free-ranging species such as the Florida panther (*Felis concolor coryi*), greater prairie-chicken (*Tympanuchus cupido pinnatus*) and koala (*Phascolarctos cinereus*) (reviewed in Hedrick & Kalinowski 2000; Keller & Waller 2002; Frankham 2003; Frankham 2010a). The loss of genetic diversity not only affects the current population and its response to stochastic events (for example: Coltman *et al*. 1999; Rijks *et al*. 2008), but also affects its ability to adapt to future changes (Lacy 1997; Frankham 2005).

Conservation biology also aims to protect the evolutionary processes which have resulted in the biological diversity we currently see (Moritz 2002). These processes can be identified by genetic and ecological analyses (Moritz 1999; Crandall *et al.* 2000; Moritz 2002). Moritz (1999) suggested the use of the evolutionary significant unit (ESU) and management unit (MU) concepts for the delineation of populations which may be regionally differentiated, and thus may require protection (Crandall *et al.* 2000, Moritz 2002). ESUs are groups of populations or MUs that are reproductively isolated and have

developed adaptive differences allowing these groups to be managed separately (Moritz 1999; Crandall *et al*. 2000). MUs are demographically independent populations where migration between populations is sufficiently low to allow for the monitoring and management of each unit independently, although limited mixing of MUs is allowed (Moritz 1999; Palsbøll *et al*. 2007). These concepts have not directly been used in the management of African elephant based on genetic data; however similar concepts, based on ecological findings, are currently being used by van Aarde & Ferreira (2009) in the development of "clusters of elephant conservation areas" which are conservation units for future elephant conservation. As a result, genetic information, together with other information known about a species, can be used to "place conservation decisions in context", so as to allow informed decision-making regarding their management (Frankham *et al*. 2002; DeSalle & Amato 2004).

Genetic diversity can be determined using a variety of molecular genetic markers, for example microsatellites, restriction fragment length polymorphisms (RFLPs), single nucleotide polymorphisms (SNPs), mitochondrial DNA (mtDNA) sequencing and major histocompatability complex II (MHCII) gene sequencing (Delport *et al*. 2001; Frankham *et al*. 2002). Microsatellites are widely used in conservation genetic studies (Bruford & Wayne 1993; Selkoe & Toonen 2006). They consist of tandem repeats of nucleotides, one to six bases pairs long (Hamada *et al*. 1982; Tautz & Renz 1984). Variations in the number of repeat units are used to designate alleles at a specific microsatellite locus (Litt & Luty 1989; Tautz 1989; Weber & May 1989; Amos *et al*. 1993). Loci are spread throughout the nuclear genome, with most di- and tetranucleotide repeats in the non-coding region of the genome (Dib *et al*. 1996; Dietrich *et al*. 1996; Metzgar *et al*. 2000; Tóth *et al*. 2000; Li *et al*. 2002). Microsatellites are co-dominant (Litt & Luty 1989; Jarne & Lagoda 1996), and inherited in a mostly Mendelian fashion (Litt & Luty 1989; Jarne & Lagoda 1996; Dakin & Avise 2004; Selkoe & Toonen 2006).

Microsatellite loci are amplified using the polymerase chain reaction (PCR, Saiki *et al*. 1985), using primers which bind to the regions flanking the tandem repeats. The flanking regions are generally well conserved within a species and sometimes between closely related species (Engel *et al*. 1996; Lowden *et al*. 2002; Barbará *et al*. 2007). Mutations can occur in the flanking regions. These mutations can prevent primers from binding during PCR amplification, resulting in so-called null alleles (Callen *et al*. 1993; Dakin & Avise 2004; Chapuis & Estoup 2007). These are nonamplifying alleles, which result in heterozygotic individuals being incorrectly identified as homozygotes or interpreted as an amplification failure; leading to incorrect estimates of population diversity and relatedness measures

(van Oosterhout *et al*. 2006; Chapuis & Estoup 2007; Girard & Angers 2008; Chybicki & Burczyk 2009; Oddou-Muratorio *et al*. 2009).

Microsatellite loci have mutation rates of 10^{-6} to 10^{-2} per locus per generation (reviewed in Schlötterer 2000; Zhang & Hewitt 2003). These high mutation rates are the result of two mechanisms: slippage during DNA replication (Levinson & Gutman 1987; Schlötterer & Tautz 1992; Tachida & Iizuka 1992), and recombination events (Harding *et al*. 1992; Richard & Paques 2000). The high levels of polymorphism observed in microsatellites (Amos *et al*. 1993) are as a result of these high mutation rates. Several models have been developed to describe microsatellite evolution (Estoup & Cornuet 1999) including: the infinite allele model (IAM, Kimura & Crow 1964), the stepwise mutation model (SMM, Kimura & Ohta 1978), the two phase model (TPM, Di Rienzo *et al*. 1994) and the *K*-allele model (KAM, Crow & Kimura 1970). Under the IAM, a mutation, which can involve any number of tandem repeats, creates a new allele previously not encountered in the population. In the SMM, a mutation causes the loss or gain of a single tandem repeat, resulting in mutations towards alleles that may already be present in the population. Under the TPM, mutations result in a gain or loss of x number of repeat units. In the KAM, K allelic states are possible, with the probability of $[\mu / (K - 1)]$ that any allele will mutate to any other $K - 1$ allelic state (μ = mutation rate). There is currently no consensus on which mutation model is ideal for microsatellites, since different data sets show better fit to different models (Estoup *et al*. 1995; reviewed in Jarne & Lagoda 1996; Estoup & Cornuet 1999; Ellegren 2004; Selkoe & Toonen 2006).

The properties of co-dominance, Mendelian inheritance, selective neutrality and high levels of polymorphism make microsatellites ideal markers for population genetic studies (Bruford & Wayne 1993; Beaumont & Bruford 1999; Zhang & Hewitt 2003; Selkoe & Toonen 2006). They have been widely used in mammals to determine genetic diversity and structuring in populations, parentage, hybridisation, bottlenecks, forensics, and phylogeography (Frankham *et al*. 2002; Zhang & Hewitt 2003; Selkoe & Toonen 2006; Sarre & Georges 2009).

The mitochondrial DNA (mtDNA) control region is another marker that is widely used in genetic studies (Avise *et al*. 1987; Harrison 1989; Avise 1994; Taberlet 1996). MtDNA is an extra-nuclear, closed-circular molecule which is maternally inherited and usually does not undergo recombination (Brown 1985; Moritz *et al*. 1987; Harrison 1989; Birky 2001; Rokas *et al*. 2003). The mitochondrial genome contains two ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes, 13 protein-coding genes and a region involved in the initiation of replication and transcription of the mtDNA molecule known as the control region (Brown 1985; Moritz *et al*. 1987). The control region is non-

coding and approximately 1000bp long, divided into two hypervariable regions and a central conserved region (Brown *et al*. 1993; Hoelzel 1993; Taberlet 1996).

The evolutionary rate over the whole mtDNA molecule is five to 10 times greater than that of single copy nuclear DNA (scnDNA, Brown *et al*. 1979; Ferris *et al*. 1983; DeSalle *et al*. 1986; Moriyama & Powell 1997). The control region is known to evolve four to five times faster than the entire mtDNA molecule (Brown *et al*. 1993). The properties of high mutation rate, maternal inheritance and non-recombination make the control region a useful marker for population genetic and evolutionary studies (Harrison 1989; Ballard & Whitlock 2004; Avise 2009). These molecular markers can potentially be used to study populations using non-invasive techniques.

Faecal DNA analysis

Since the early 1990's, an increasing number of molecular population genetic studies of free-ranging animal populations have reported the use of non-invasive DNA sampling techniques (see amongst others: Kohn & Wayne 1997; Taberlet *et al.* 1999; Beja-Pereira *et al*. 2009). These techniques have included obtaining DNA from shed hairs (Taberlet & Bouvet 1992; Taberlet *et al.* 1997; Marshall & Ritland 2002; Clifford *et al*. 2004; Roon *et al*. 2005), shed feathers (Segelbacher 2002; Petersen *et al*. 2003; Rudnick *et al*. 2005), urine (Yokota *et al.* 1998; Valiere & Taberlet 2000), sloughed/shed skin (Palsbøll *et al*. 1997; Elphinestone *et al*. 2003; Swanson *et al*. 2006), and faeces (Kohn *et al.* 1995; Reed *et al.* 1997; Kohn *et al.* 1999; Okello *et al*. 2005; Gobush *et al*. 2009), where the animal itself is not disturbed (Taberlet *et al*. 1999). Of these techniques, faecal samples are relatively easy to obtain in the wild (Beja-Pereira *et al*. 2009).

Intestinal epithelial cells are shed during defecation, therefore specific DNA sequences can be amplified from these cells using PCR (Saiki *et al*. 1985) (Kohn & Wayne 1997; Hofreiter *et al*. 2001; Morin *et al*. 2001). In this way the genetic structure, demography, life history, population subdivision, food habits, reproduction and sex ratios of a species can be assessed (Kohn & Wayne 1997; Reed *et al.* 1997; Taberlet *et al*. 1997; Kohn *et al*. 1999; Ernest *et al*. 2000; Farrel *et al*. 2000; Vidya *et al*. 2005a; Piggott *et al.* 2006; Gobush *et al*. 2009); adding to information regarding a species' biology, particularly for elusive animals or species of conservation concern. These methods are also useful where capturing individuals is undesirable, such as in behavioural studies or in small endangered populations (Tikel *et al*. 1996; Kohn & Wayne 1997; Taberlet *et al.* 1999; Ernest *et al*. 2000; O'Ryan *et al*. 2001; Wehausen *et al*. 2004; Piggott *et al*. 2006; Eggert *et al*. 2008).

Unfortunately DNA obtained via non-invasive sampling is of both low quantity and quality which leads to various problems (Beja-Pereira *et al*. 2009). These include: DNA contamination from other sources of DNA, poor DNA amplification due to the presence of PCR inhibitors, possibility of genotyping errors, and DNA degradation (Taberlet *et al*. 1996; Kohn & Wayne 1997; Taberlet *et al*. 1997; Goosens *et al*. 1998; Taberlet *et al.* 1999; Bradley & Vigilant 2002; Murphy *et al*. 2002; Creel *et al*. 2003; Fernando *et al*. 2003; Murphy *et al*. 2003; Eggert *et al*. 2005; Broquet *et al*. 2007; Beja-Pereira *et al*. 2009).

DNA from shed epithelial cells are likely to decay due to their exposure to the environment (Kohn & Wayne 1997). The repair mechanisms found in living cells do not function in these sloughed 'dead' cells; therefore the DNA is exposed to both hydrolytic and oxidative decomposition and is spontaneously degraded at moderate temperatures, such as those that would be present in the field (Lindahl 1993). Continued ambient moisture, exposure to sunlight and continual bacterial degradation will result in DNA that is degraded to such an extent that it can no longer be used in PCR amplification (Foran *et al*. 1997; Reed *et al*. 1997; Hofreiter *et al.* 2001; Fernando *et al*. 2003).

The storage of dung samples is extremely important, so as to counteract environmental effects on DNA (Taberlet *et al*. 1999; Beja-Pereira *et al*. 2009). The effectiveness of various storage methods on the success of obtaining amplifiable DNA has been compared in previous studies (Wasser *et al*. 1997; Frantzen *et al*. 1998; Murphy *et al*. 2002; Fernando *et al*. 2003). Dung can be either be stored dry (for example: air dried, silica dried) or in a liquid storage medium (for example: DMSO/saturated NaCl solution, ethanol). Although the storage of elephant dung in DMSO/saturated NaCl solution has been used successfully in other elephant studies (Nyakaana & Arctander 1999; Eggert *et al*. 2002; Nyakaana *et al*. 2002; Fernando *et al*. 2003; Okello *et al*. 2005; Eggert *et al*. 2008; Gobush *et al*. 2009), one should heed Frantzen *et al*.'s (1998) advice that "results will possibly vary according to ... ecological conditions at the study site".

DNA that is present in numerous copies within a single cell, such as animal mtDNA, is more likely to survive DNA decay (Hofreiter *et al.* 2001). Therefore, good quality mtDNA can be obtained from faecal samples for further analysis (Kohn & Wayne 1997; Hofreiter *et al.* 2001). However, single copy nuclear DNA, such as microsatellites, is degraded into small fragments and is present in very low quantities (Taberlet *et al.* 1996; Taberlet *et al.* 1999). As a result: (1) PCR amplification may not be possible, (2) a PCR product with the correct genotype may be obtained or (3) a PCR product with the incorrect genotype may be obtained (Taberlet *et al.* 1999). Incorrect genotyping may be due to 'allelic dropout' or due to the generation of 'false alleles'. 'Allelic dropout' generally occurs due to stochastic

events during template DNA pipetting, such that only a single allelic copy is detected in a true heterozygote. Furthermore, 'allelic dropout' may occur since DNA degradation has taken place to such an extent that only one allelic copy can be amplified (Kohn & Wayne 1997; Taberlet *et al.* 1999; Hofreiter *et al.* 2001). 'False alleles' refer to the generation of an extra allele when the individual is a true homozygote or the generation of three alleles when the individual is a true heterozygote. 'False alleles' are caused by PCR amplification artefacts due to slippage during the first few PCR cycles, especially when amplifying dinucleotide microsatellites (Taberlet *et al.* 1996; Goosens *et al.* 1998). To overcome these genotyping problems, Taberlet *et al.* (1996) suggested repeating the amplification of a specific locus for a single DNA sample between three and seven times until a homozygote or heterozygote can be confirmed. Many other researchers (see for example Kohn *et al.* 1995; Ernest *et al.* 2000) have used repeated extractions from a single faecal sample, since one extraction may produce enough DNA for further PCR amplification and another extraction from the same sample may produce no amplification product at all. These guidelines are extremely useful in preventing genotyping errors, but they lead to increased cost and time in obtaining results.

Because PCR can amplify minute quantities of DNA, the risk of contamination of faecal DNA extracts with non-target DNA is large (Kohn & Wayne 1997; Taberlet *et al.* 1999). To overcome this problem several guidelines are generally followed: the physical isolation of areas for DNA extraction and PCR preparation, the use of disposable gloves, the use of aerosol resistant filter pipette tips, as well as the use of positive and negative controls during DNA extraction and PCR preparation (Kwok & Higuchi 1989; Taberlet *et al.* 1996; Reed *et al.* 1997; Wasser *et al.* 1997; Kohn *et al.* 1999; Ernest *et al.* 2000; Segelbacher 2002; Eggert *et al*. 2005; Beja-Pereira *et al*. 2009). These methods again result in increased expense (Taberlet *et al*. 1999; Beja-Pereira *et al*. 2009).

PCR inhibitors co-extracted with DNA further reduce amplification rates (Beja-Pereira *et al*. 2009). These PCR inhibitors may be of plant origin from the animal's diet (O'Ryan *et al.* 2001), may be due to bilirubin and bile salts (Deuter *et al.* 1995), bacteria (Beja-Pereira *et al*. 2009), or from other unknown inhibitors (Pääbo 1990). Various chemicals have been suggested to overcome inhibition, including: bovine serum albumin (BSA, Pääbo 1990), hexadecyltrimehylamonium bromide (CTAB, Constable *et al.* 1995), or polyvinylpyrrolidone (PVP, O'Ryan *et al.* 2001). New DNA extraction techniques, such as the adsorption of nucleic acids onto silica beads (Höss & Pääbo 1993), further decrease the inhibition of *Taq* polymerase during PCR.

The DNA extraction technique is one of the most crucial steps in any non-invasive genotyping study. The ideal DNA sample would be one of high quality and quantity, with the absence of any PCR

inhibitors. There are no standard protocols for DNA extraction from faeces (Eggert *et al*. 2005; Beja-Pereira *et al*. 2009), even for elephant samples (see Eggert *et al*. 2002; Nyakaana *et al*. 2002; Fernando *et al*. 2003; Okello *et al*. 2005; Gobush *et al*. 2009). Newer DNA extraction techniques which include a purification step, such as Boom *et al*.'s (1990) method where DNA is adsorbed onto silica beads, have shown much promise in faecal DNA studies (Eggert *et al.* 2005; Beja-Pereira *et al*. 2009).

Why elephant dung?

Previous genetic studies on African elephant have obtained DNA from tissue samples of culled animals (Osterhoff *et al*. 1974; Georgiadis *et al*. 1994; Coetzee *et al*. 1999; Whitehouse & Harley 2001), tissue samples obtained by biopsy darting (Karesh *et al*. 1987; Georgiadis *et al*. 1994; Nyakaana & Arctander 1999; Nyakaana *et al*. 2001; Roca *et al*. 2001; Whitehouse & Harley 2001; Comstock *et al*. 2002; Nyakaana *et al*. 2002), or tissue/blood samples from animals immobilised for veterinary attention or for translocation (Osterhoff *et al*. 1974; Georgiadis *et al*. 1994). Behavioural studies of elephant populations in small game reserves in South Africa (Burke *et al.* 2008) make the use of conventional methods for obtaining DNA samples, such as biopsy darting (Karesh *et al*. 1987), undesirable. However, there are studies that have successfully used dung as a source of DNA (Nyakaana & Arctander 1999; Nyakaana *et al*. 2002; Eggert *et al*. 2002; Okello *et al*. 2005, Eggert *et al*. 2008, Archie *et al.* 2008, Okello *et al*. 2008a; Gobush *et al*. 2009), with Okello *et al*. (2005) reporting up to a 98% success rate in correctly genotyping 202 individual elephants. The ease with which dung samples can be obtained and the possibility of sampling large numbers of individuals in a population make non-invasive sampling a potentially valuable technique to investigate for further studies. Before a large-scale molecular study using faecal DNA can be carried out, a pilot study to determine the feasibility of faecal DNA for a population genetic study is critical (Taberlet *et al*. 1999; Beja-Perreira *et al*. 2009).

Elephant genetic studies

Since the development of molecular genetic techniques, elephants (both African and Asian (*Elephas maximus*) have been studied using a variety of markers ranging from allozymes, mtDNA restriction fragment length polymorphisms (RFLPs), microsatellites to DNA sequencing of various loci. These markers have been used to study elephants at various scales, from local herds to regional and continent-wide scales (Sukumar 2003).

Genetic studies on African elephant populations have investigated population structure and genetic diversity (Osterhoff *et al.* 1972; Osterhoff *et al.* 1974; Essop *et al.* 1996; Coetzee *et al.* 1999;

Nyakaana & Arctander 1999; Nyakaana *et al*. 2001; Nyakaana *et al.* 2002; Archie *et al*. 2006; Archie *et al*. 2007; Ishengoma *et al*. 2007; Archie *et al*. 2008; Eggert *et al*. 2008; Okello *et al*. 2008a, b; Gobush *et al*. 2009), parentage (Whitehouse & Harley 2001; Archie *et al*. 2007; Hollister-Smith *et al*. 2007; Ishengoma *et al*. 2007), bottlenecks (Whitehouse & Harley 2001), forensics (Wasser *et al*. 2004; Wasser *et al*. 2007), phylogeography (Georgiadis *et al.* 1994; Siegismund & Arctander 1995; Comstock *et al*. 2002; Eggert *et al*. 2002), and the savanna/forest elephant species debate (Roca *et al*. 2001; Debruyne 2005; Roca & O'Brien 2005; Johnson *et al.* 2007; Roca *et al*. 2007). Many of these studies have tried to provide guidelines for the management of the species as a whole, however, the difficulty in identifying the exact number of elephant 'taxa' present in Africa has prevented this (Eggert *et al*. 2002; Nyakaana *et al*. 2002). At the regional and population level, studies have warned against the potential effects of a loss in genetic diversity due to genetic drift, founder effect, inbreeding/inbreeding depression, or the disruption of social groups (due to poaching); advocating the use of genetic data when developing population management plans (Nyakaana *et al*. 2001; Whitehouse & Harley 2001; Archie *et al*. 2008; Okello *et al*. 2008).

Initial studies used protein electrophoresis to determine genetic variation in populations. Studies on KNP elephants (Osterhoff *et al.* 1972; Osterhoff *et al.* 1974) looked at haemoglobins, serum transferrins and other enzymes and found almost no variation, leading to the conclusion that the KNP elephants are genetically homogeneous. A study investigating the genetic variation in the KNP elephants culled in 1992 and 1993 used 25 protein loci (Coetzee *et al.* 1999), of which only six loci showed polymorphism. Results showed that there was a decrease in heterozygosity values from adults (3.7%) to juveniles (2.1%). Individuals in the northern part of KNP had lower heterozygosity levels (3.3 %) than individuals in the central part of KNP (4.7%) giving an average of 4% for both these areas. In a study comparing both African and Asian elephants (Drysdale & Florkiewicz 1989), only five out of 23 protein loci were found to be polymorphic. Mean heterozygosity was estimated at 8% for the African elephants and 7.2% for the Asian elephants.

The advent of PCR allows the amplification of targeted DNA sequences *in vitro* quickly and efficiently. Initial studies involving mtDNA determined haplotypes by RFLPs of amplified regions. Georgiadis *et al.* (1994) found only 10 mtDNA haplotypes of which two were widespread throughout eastern and southern Africa. Haplotypes representing exceptionally divergent mitochondrial haplogroups were found to co-occur within distant savanna elephant populations. Furthermore, marked subdivision was identified at the continental level whereas there was no significant differentiation at the regional level. These results can be explained by the fact that elephant populations are characterised by protracted but intermittent gene flow within a subdivided population.

Siegismund & Arctander (1995) reanalysed the above data using a procedure for detecting geographic subdivision developed by Hudson *et al.* (1992). Similarly they found that there was significant variation at the continental level, but in contrast to Georgiadis *et al.* (1994), they found that African elephant populations are subdivided on a regional level due to either a single or a small number of populations having diverged significantly from each other. A similar study was conducted on elephants from KNP and AENP (Essop *et al*. 1996) in which mtDNA was digested with 12 restriction enzymes. Sequence diversity was estimated at 0.27% for the KNP population and 0.38% for the combined populations. The KNP population had two variants at three restriction enzymes whereas the Addo population was completely monomorphic. In addition, all of Addo's restriction patterns were present in the KNP population indicating that gene flow occurred historically between these two populations and therefore Addo is a genetic subset of the larger KNP population.

Current mtDNA studies involve the analyses of DNA sequences of various regions in mtDNA, for example the control region and cyt *b* gene. Studies of the mtDNA control region have investigated diversity at the continental and inter-regional level (Eggert *et al*. 2002; Nyakaana *et al*. 2002; Johnson *et al*. 2007). On a continental level elephant mtDNA control region had an average nucleotide diversity of 3.0% (lower than other large African mammals, Nyakaana & Arctander 1999) and haplotype diversity of 0.99 (Johnson *et al*. 2007). Highly divergent mtDNA haplotypes, not necessarily control region, have been found to coexist in eastern and southern populations (Tiedemann *et al*. 1998; Nyakaana & Arctander 1999; Eggert *et al*. 2002; Nyakaana *et al*. 2002). Two hypotheses have been proposed for the distribution of haplotypes between regions: (1) "large long-term effective population sizes" which resulted in incomplete lineage sorting and (2) "recent secondary population admixture due to range expansion from refugia" (Georgiadis *et al*. 1994; Eggert *et al*. 2002; Nyakaana *et al*. 2002). Georgiadis *et al*. (1994) argued that there is no evidence of geographical barriers that could have isolated populations for a sufficient amount of time leading to such high levels of divergence, thus supporting the first hypothesis. However, Nyakaana *et al*. (2002) highlighted that there is no evidence of large census populations having existed in Africa, rather supporting the second hypothesis. Eggert *et al*. (2002) and phylogeographic studies on other large African mammals (reviewed in Hewitt 2004); including kob (*Kobus kob*, Lorenzen *et al*. 2007), giraffe (*Giraffa camelopardalis*, Brown *et al*. 2007), and gorilla (*Gorilla gorilla* and *Gorilla beringei*, Ackerman & Bishop 2009); provide further support for the refugial hypothesis. Phylogenetic network analyses of mtDNA control region at the continental level have identified three (Nyakaana *et al*. 2002) to four (Eggert *et al*. 2002; Johnson *et al.* 2007) haplogroups among African elephants. Each 'savanna' haplogroup identified in these three studies found highly divergent mtDNA haplotypes to coexist in eastern and southern African populations. However, despite Johnson *et al.* (2007) having added

haplotypes from central African forest areas, the limited number of samples from the entire range of the African elephant may result in incorrect assumptions of molecular structure (Debruyne 2005).

Nuclear DNA variation has been studied with the use of microsatellites. Currently 43 microsatellite primers have been published for African elephants (Nyakaana & Arctander 1998, Comstock *et al*. 2000, Eggert *et al*. 2000, Comstock *et al*. 2002, Archie *et al*. 2003, Nyakaana *et al*. 2005), and have been successfully used in both 'savanna' and 'forest' African elephants (Whitehouse & Harley 2001; Comstock *et al*. 2002; Eggert *et al*. 2002; Wasser *et al*. 2004; Eggert *et al*. 2008; Ishengoma *et al*. 2007; Okello *et al*. 2008a; Gobush *et al*. 2009) as well as in Asian elephants (Fernando *et al*. 2003; Vidya *et al*. 2005a, b; Fickel *et al*. 2007; Vidya *et al*. 2007).

Nyakaana and Arctander's (1999) study on three Ugandan populations was the first study to combine both microsatellite and mtDNA data. Analysis of population subdivision showed significant subdivision at the mtDNA level where microsatellite data showed weak or no subdivision between populations. Similarly, mtDNA data of Kenyan populations showed significant subdivision at the regional level, whereas microsatellite data showed significant differentiation among populations within regions (Okello *et al*. 2008a). The difference in genetic structure has been explained in terms of male-biased gene flow between populations which results in the homogenisation of nuclear alleles and the restriction of maternally inherited mtDNA alleles to specific localities (Nyakaana & Arctander 1999), or due to cytonuclear genomic dissociation of the mitochondrial and nuclear genomes (this hypothesis has been used in the savannna/forest elephant debate, Roca *et al*. 2005; Roca *et al*. 2007). Elephant social structure can have an effect on microsatellite variation. Since related females are found in family groups and mating involves females and unrelated males (Skinner & Chimimba 2005), one can expect heterozygote excess (Nyakaana *et al*. 2001). This hypothesis was confirmed by significant heterozygote excess in Ugandan populations (Nyakaana & Arctander 1999), whereas a breakdown in social behaviour was detected in the Queen Elizabeth National Park where significant heterozygote excess was only found in one family group and three family groups had more than one mtDNA haplotype per family group (Nyakaana *et al*. 2001).

The KNP, the AENP and the Knysna elephant populations are the only South African elephant populations for which genetic studies using microsatellites and/or mtDNA control region have been published (Whitehouse & Harley 2001; Comstock *et al*. 2002; Eggert *et al*. 2002; Nyakaana *et al*. 2002; Wasser *et al*. 2004; Wasser *et al*. 2007; Eggert *et al*. 2008). A microsatellite study on the genetic diversity of the Addo population, in comparison to the KNP population, found that there was a significant reduction in the genetic diversity in this population, both in allele numbers and

heterozygosity (Whitehouse & Harley 2001). Whitehouse and Harley (2001) observed high levels of genetic differentiation between the Addo and Kruger populations, suggesting rapid genetic drift of the Addo population due to its small population size (11 individuals (Trollope 1931) after the proclamation of the park in 1931). A study on the Knysna elephants detected five individuals in a population that was previously believed to be extinct. Genetic diversity of these individuals was higher than that of Addo, but similar to that of Kruger. Furthermore, a single mtDNA control region haplotype was shared among all five individuals and this haplotype matches one previously found in the Addo population (Eggert *et al*. 2008). The Kruger population has lower genetic diversity than three Ugandan populations for the same microsatellite loci (Nyakaana & Arctander 1999; Whitehouse & Harley 2001), and the expected heterozygosities of southern African populations were lower than that of eastern African populations studied (Nyakaana *et al*. 2002). Despite eastern African elephant populations having suffered severe declines in numbers during the 1970's and 1980's due to ivory poaching (Ottichilo *et al*. 1987) which affected their genetic diversity, southern African elephant populations suffered severely during the eighteenth and nineteenth centuries due to hunting and poaching (Hall-Martin 1992; Skinner & Chimimba 2005) which resulted in a "lasting loss of genetic diversity" in the region (Whitehouse & Harley 2001). In contrast to these studies, Comstock *et al*.'s (2002) study found that southern and eastern African populations had similar observed heterozygosities and that southern African populations had more alleles than eastern African populations.

The genetic diversity of reintroduced elephant populations has to date not been assessed. The information obtained in a genetic study of these populations can be used to aid in future management decisions in order to reduce potential genetic threats.

Study objectives

The aim of this study was to investigate demographic and genetic factors that may potentially increase extinction risk in reintroduced elephant populations in South Africa. Knowledge of the effects of these factors allows the development of management plans that reduce extinction risk.

The following questions were asked:

1. Which demographic factors influenced the growth of the Pilanesberg National Park elephant population from 1989 to 2003?
2. Which demographic factors could have the greatest effect on future population growth?

3. Can elephant dung be used as a reliable source of DNA for population genetic studies of South African elephants?
4. What is the genetic diversity in the reintroduced populations of Pilanesberg National Park, Madikwe Game Reserve, Hluhluwe-iMfolozi Park and Phinda Private Game Reserve? How does this diversity compare to the population in the KNP, the source of many of the founder individuals, and the AENP, a population known to have suffered a severe bottleneck?
5. What is the genetic diversity of southern African elephant populations and how does this compare to that of eastern African populations?

The answers to these questions will provide insights into how demographic and genetic factors can influence small populations of elephants. This insight can then be used in the development of future management plans for reintroduced elephant populations.

Dissertation outline

Chapter 1: General introduction
Introduction and literature review.

Chapter 2: Modelling a small population of elephants – An evaluation of three models
Three population models, namely: a Leslie matrix model, a stochastic age and stage-structured model based on Wu & Botkin's (1980) model, and VORTEX a stochastic individual-based model, were used to simulate the historical growth of the Pilanesberg National Park elephant population, as an example of an introduced population of elephants. Future projections, including harvesting as a management action, were also investigated using these models.

Chapter 3: Non-invasive DNA sampling from elephant dung – A pilot study
A pilot study using dung from Zoo elephants was conducted to determine the feasibility of using faeces as a source of DNA. Various faecal storage and faecal DNA extraction methods were critically evaluated for efficiency and repeatability under local conditions. The quality and quantity of DNA obtained was evaluated by PCR amplification of mtDNA and microsatellite loci.

Chapter 4: Conservation genetics of small populations of elephants in South Africa
Microsatellite diversity of translocated elephant populations are presented and compared to that of populations in the Kruger National Park, the source of many of the founder individuals, and the Addo Elephant National Park, a population known to have suffered a severe bottleneck. Microsatellite and

mtDNA control region diversity within the southern African region was investigated to determine whether genetic diversity had been lost by heavily managed populations in South Africa. The effects of historical and recent population reductions on continental-level genetic diversity, and the presence of two distinct savanna mtDNA control region haplogroups were explored.

Chapter 5: Conclusion

Chapter 2[1]

Modelling a small population of elephants - An evaluation of three models

Abstract

Elephant have been introduced into about 60 small (< 1000 km^2) fenced reserves in South Africa, resulting in small populations with restricted immigration and emigration. Due to the demographic structure of the founding populations, many of these populations experience growth rates greater than the long-term maximum of 7% per annum. Understanding these high growth rates can aid in future management. A modelling approach, comparing three different models, was used to determine the demographic factors that influenced the historical growth of the Pilanesberg National Park elephant population, one of the oldest and largest introduced populations. Future projections investigated demographic factors affecting growth, as well as the effects of harvesting as a management intervention. An annual population growth rate of 12.1% was calculated for this population over the period 1989 to 2003. The mean intercalving interval and mean age at first calving were at the lower range of values compared to well-established populations in southern and eastern Africa. Future projections (2003-2023) indicated population growth rates of between 8.1 and 8.5%. Intercalving interval was the most sensitive demographic factor in future projections. Harvesting of adult males was found to be a very effective short-term option for managing population growth. Despite stochastic models being more representative of reality, further studies should be conducted on the effectiveness of the models used for simulating small populations of elephants.

[1] This chapter will be shortened for submission to Oryx. Authorship will be as follows: Alves Ferreira MA, Bloomer P and Slotow R.

Introduction

Many of the conservation areas outside national parks in South Africa consist of small areas of land, generally less than 1000 km^2, which were reclaimed from areas that were previously used for agriculture (Hall-Martin 1992). Most of these areas were repopulated with animals that historically occurred in these areas, resulting in the introduction of small populations of animals into small areas of land (e.g. Slotow *et al.*, 2005; Gusset *et al.*, 2008; Kettles & Slotow, 2009; Morgan *et al.*, 2009). Due to these areas being fenced-in, many function essentially as islands with restricted immigration or emigration (Slotow *et al.* 2005). The only factors affecting the demography of these populations therefore involve births and deaths, as well as artificial introductions or removals.

Well over 800 elephants (*Loxodonta africana*) have been introduced into 58 conservation areas since 1979 (Garaï *et al.* 2004). In 2003 the largest of these reintroduced populations numbered 387 individuals in a fenced area of approximately 900 km^2 (Hluhluwe-iMfolozi Park, Mackey *et al.* 2006) and one of the smallest populations numbered 10 individuals in a fenced area of approximately 80 km^2 (Mabula Game Reserve, Mackey *et al.* 2006). These population sizes are in stark contrast to more 'natural' elephant populations (Slotow *et al.* 2005; Mackey *et al.* 2006) such as the Kruger National Park population, estimated at 12 400 in 2006 (Blanc *et al.* 2007).

The founding populations for many small reserves consisted of small numbers of juveniles with female-skewed sex ratios (Whyte 2001; Garaï *et al.* 2004; Slotow *et al.* 2005; Mackey *et al.* 2006). Many of these populations now consist of young adults resulting in the problem of high growth rates and almost zero mortalities due to effective protection (Slotow *et al.* 2005; Mackey *et al.* 2006). Groups of similarly aged individuals will reach maturity at the same time, resulting in 'waves of births' causing an increase in population growth (Dominy *et al.* 1998; Slotow *et al.* 2005; Mackey *et al.* 2006). The female-skewed sex ratio moreover results in a high growth potential (Slotow *et al.* 2005; Mackey *et al.* 2006).

The 'abnormal' population structure of these reintroduced populations has led to growth rates greater than Calef's theoretical maximum population growth rate of 7% per annum (Calef 1988). As a result, stochastic factors such as demographic, environmental and genetic stochasticity as well as natural catastrophes can potentially affect the viability of these populations (Shaffer 1981; Lande 1993; Caughley 1994; Lacy 2000a; Lande *et al.* 2003; Melbourne & Hastings 2008). Demographic stochasticity refers to the random variation in the number of births and deaths as well as variation in sex ratios (Caughley 1994). Environmental stochasticity is the result of temporal fluctuations in

weather conditions (Shaffer 1981; Lacy 2000a; Lande *et al*. 2003). Genetic problems in small populations may be due to inbreeding or the loss of genetic diversity (Hartl 1988; Lacy 1997; Meffe & Carroll 1997; Delport *et al*. 2001; Frankham *et al.* 2002; Frankham 2010a). All these factors are not necessarily mutually exclusive, and may at times influence each other or even compound the effect of a single factor (Lacy 2000a).

Due to the unexpected growth rates of these small populations of reintroduced elephant there is a need to understand the demographic factors that have affected population growth (Slotow *et al*. 2005; Mackey *et al*. 2006). In this context, modelling is an ideal tool for analysing various aspects of a population (Starfield & Bleloch 1991). Similarly, several management questions can be simulated and their likely consequences determined (Owen-Smith *et al*. 2006). Furthermore, future projections of these populations can be used to investigate conservation concerns and can aid in making well-informed management decisions. Various modelling methods have been developed for the analysis of single-species populations; these include deterministic, stochastic, age-structured and individual-based models (Starfield & Bleloch 1991; Uchmański & Grimm 1996).

The Leslie matrix model is an example of a deterministic age-structured model (Jeffers 1982; Caswell 2001; Gallego 2003). This model requires very little data from the population and is relatively simple mathematically (Jeffers 1982; Allendorf & Ryman 2002). Since this is a deterministic model, stochastic factors that affect a population are ignored (Jeffers 1982; Caswell 2001; Allendorf & Ryman 2002; Gallego 2003), density feedback is not included (Gallego 2003), and individual variability within each age-class is not considered (Uchmański & Grimm 1996; Allendorf & Ryman 2002).

Stochastic models are considered more appropriate for long-lived species with overlapping generations (Sukumar 2003). The first stochastic model designed specifically for studying the dynamics of African elephants was presented by Wu and Botkin (1980). This model is a discrete-time, stochastic model where a female's biological state at each age is considered (Wu & Botkin 1980; Gallego 2003). Compared to the Leslie model, this model is more realistic (Wu & Botkin 1980; Gallego 2003; Sukumar 2003); however more detailed data is required particularly for females (Wu & Botkin 1980; Gallego 2003).

VORTEX is a stochastic population modelling programme using individual-based Monte Carlo simulation (Lacy 1993). Population dynamics are modelled as discrete, sequential events that occur according to defined probabilities (Lacy 1993). This model includes demographic stochasticity,

environmental stochasticity, catastrophes as well as genetic stochasticity (Lacy 1993; Lacy 2000b). However, this model requires more population data than the Wu & Botkin (1980) model and females are modelled per age class and not by biological state. Increasing reserve sizes, immunocontraception, distortion of sex ratios in order to decrease growth potential, translocation, culling and hunting have been suggested for the management of elephant populations (Slotow *et al*. 2005; Owen-Smith *et al*. 2006). In VORTEX the harvesting option can be used to mimic hunting, culling and the removal of animals for translocation (Miller & Lacy 2003).

The aim of this chapter was to compare how well three models namely a Leslie matrix model, Wu and Botkin's (1980) discrete-time, stochastic population model and VORTEX can simulate the growth of the Pilanesberg National Park elephant population as an example of a small reintroduced population of elephants in South Africa. The historical growth of the population from 1989 to 2003 was examined and the demographic factors that influenced the population's rate of increase were determined. These three models were also used to investigate the demographic factors that could have the greatest effect on future population growth. The potential effect of harvesting, as a management action, was investigated.

Materials and methods

Study area and Population history

Pilanesberg National Park (PNP; 25°08'- 25°22'S; 26°57'- 27°13'E) is situated in the remains of an extinct volcano in the North-West Province of South Africa (Fig. 1). The park comprises 500 km^2 of fenced-in hilly savanna terrain. The habitat consists of *Acacia* and broad-leaf bushveld which ranges from thickets to open grassland patches. Water is readily available in the park due to a major river system (Mankwe River) that runs in a south-easterly direction through the central part of the park, as well as a large dam in the centre of the park and several smaller dams scattered throughout the park. Annual rainfall is approximately 630 mm, and falls mainly in summer. Winters are cold (minimum temperature 1°-5°C) and summers are very hot (maximum temperature 28°-31°C) (Slotow & van Dyk 2001; van Dyk & Slotow 2003).

Since the proclamation of the park in 1979, numerous animal species, including elephant, were reintroduced into PNP. Five elephants were initially introduced from Addo Elephant National Park (AENP) in 1979, but three individuals died shortly after the translocation, one was returned to AENP, leaving only one young male in PNP (Anderson 1994). A further 18 infants were introduced in 1981 from Kruger National Park (KNP) (Anderson 1994). In 1982 two tame, circus-trained adult females (originally from KNP) were introduced and they assumed the role of matriarchs (Bosman & Hall-

Martin 1986). Further introductions from KNP included 26 and 36 individuals in 1983 and 1993 respectively, and the first calf was born in 1989 (Anderson 1994; Slotow & van Dyk 2001). In the early 1990's young elephant males were found to be a major cause of mortality of white rhino (*Ceratotherium simum*, Slotow & van Dyk 2001). Males were found to be entering musth (a state of heightened sexual and aggressive activity in male elephants, Poole & Moss 1981) at a much earlier age than is found in natural elephant populations. In 1998 six adult bulls were introduced into the park to stabilise the bull hierarchy in PNP (Slotow *et al*. 2000; Slotow & van Dyk 2001). In December 2005 the population totalled 136 individuals of which 34 were independent bulls and 102 were breeding herd individuals in 18 family groups (Woolley *et al.* 2008b).

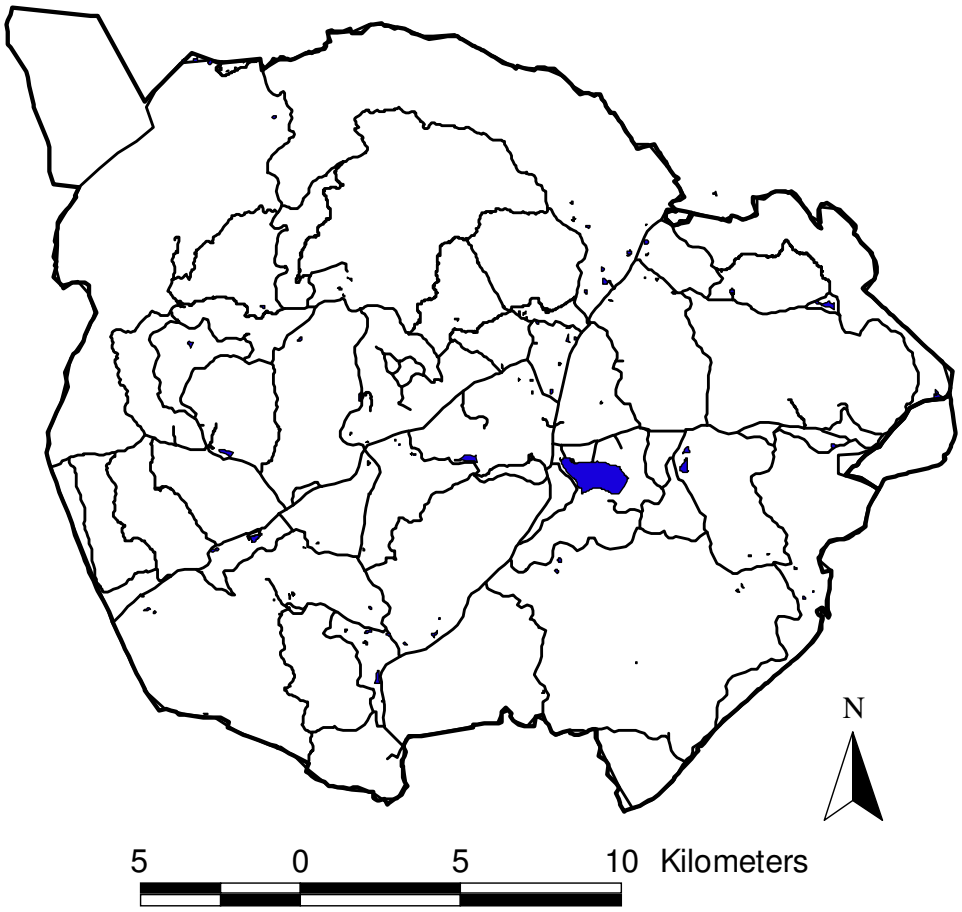

Figure 1 Map of Pilanesberg National Park, North-West Province, South Africa showing roads (including management tracks, in black), and dams (in blue).

Population Data

Data were obtained, from management records, detailing the number of individuals introduced, individuals that died as a result of natural causes, or those removed due to hunting (Burke *et al*. 2008). In 1998 the Pilanesberg elephant project was started to study several ecological and behavioural aspects of the elephant population in PNP. During this project all the adult elephants in PNP were identified and an identikit for each animal compiled. These identikits use unique ear, trunk, tusk, and tail markings to identify individual adults. Detailed population breakdowns (including age structure and sex ratio) were only available for 1998 and 2001 (van Niekerk *et al.* 2001), and for the period 2002-2003 (Burke 2005). See Appendix 1 for a summary of the data.

Retrospective Modelling

Population data were analysed to determine the growth rates of the population over several time spans. Observed annual exponential rates of increase (r) were determined by taking the natural logarithms of the population estimates and fitting a linear regression to the data points, with the slope of the regression line equal to the observed rate of increase; i.e. $r = \ln(N_{t+1}/N_t)$, where N = population size and t = time (Caughley & Sinclair, 1994). Population growth was determined as % population growth = $(e^r-1) \times 100$ (Caughley & Sinclair, 1994). The population was modelled over two time periods, namely 1989 to 1998 (first births to beginning of the Pilanesberg elephant project, data not accurate) and 1998 to 2003 (accurate data from Pilanesberg elephant project). The observed rate of increase (r) over the full study period (1989 to 2003) was also determined. Rates of increase before 1989 were excluded since, although introductions were taking place, there were no births. The data produced by the models were analysed using the linear regression method, as described previously, to determine the 'modelled' rate of increase. T-tests comparing two slopes (Zar 1996) were conducted to determine whether the 'modelled' rates of increase were significantly different from the observed rates of increase.

Information on the population (as summarised in Appendix 1) was used to reconstruct the population in 1989 and 1998 (see Appendices 2a-b) by working in reverse chronological order as well as combining this with known population breakdowns, reintroductions and deaths or hunts. To simulate the addition of elephants to a population, Gallego (2003) suggests simulating the population up to the year of supplementation, modifying the population breakdown obtained to include the additional individuals and using this new population breakdown as the initial population for the remaining years to be simulated. This method is however tedious; I therefore made certain assumptions regarding the 1989 population: individuals introduced in 1981 were approximately two to three years old at introduction, and would be approximately 10 to 11 years old in 1989; individuals introduced in 1983

were approximately four to nine years old, and would be approximately 10 to 15 years old in 1989; individuals introduced in 1993 (approximately six to 10 years old at introduction) were included into the 1989 population at which time they were assumed to be approximately two to six years old (R. Slotow, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication). Because of this last assumption, the observed rate of increase was recalculated by adding the 1993 individuals to the 1989 population, as well as excluding the six adult bulls introduced in 1998 from the 1998 population.

The reconstructed 1989 population was simulated for nine years, and the reconstructed 1998 population was simulated for five years, using three population models. The first two models, a Leslie matrix model and a modification of the Wu and Botkin (1980) discrete-time stochastic model, were run using the computer programme Simulele, version March 2003, developed by Gallego (2003). The third model, VORTEX version 9.13 (Lacy *et al.* 2003), is an independent computer programme. The default parameters used by both programmes, given in Table 1, were determined from the literature (Laws 1969; Hanks 1972; Sherry 1975; Kerr 1978; Jachmann 1986; Viljoen 1988; Moss 2001; Whitehouse 2001; Whyte 2001), as well as from the PNP population. In VORTEX, when a population reaches its designated carrying capacity additional mortality is imposed on all age and sex classes, such that the population is reduced to below this limit (Miller & Lacy 2003). The ecological carrying capacity of PNP for elephants is unknown (R. Slotow, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication), and to date no South African elephant population has been found to be regulated by density dependence (van Jaarsveld *et al*. 1999; Whitehouse 2001; Gough & Kerley 2006). To minimise the effects of an imposed ecological mortality, carrying capacity was set to 500, well above the modelled population size.

Various values for population parameters were simulated to determine which factors most greatly affected the observed rate of increase in the PNP population. These parameters included: intercalving interval, age at first calving, sex ratios and death rates. Yearly population size was estimated by determining the mean population size projected from 100 simulation replicates.

Future population growth

Using the demographic parameters obtained for the PNP population over the period 1998-2003, the 2003 population was projected for a further 20 years using the three models described previously. The population breakdown determined in 2003 (T. Burke, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication) was used as the starting point (Appendix 2c). A sex ratio of 50% females : 50% males was imposed on calves for which the sex was

unknown. The mean calving interval was set at three years and the mean age of a cow giving birth to her first calf was set at 12 years. In VORTEX the carrying capacity was set to 1000 individuals, a value well above the largest population size projected.

Sensitivity analyses were run on the initial results by varying the intercalving interval, age at first calving and death rate. Since the population structure cannot be changed from within the Simulele programme, only VORTEX was used to simulate the effects of the removal of entire herds or adult males from the population. VORTEX was further used to determine the effects of various levels of inbreeding depression. These analyses were used to determine what effect changes in the above-mentioned variables might have on the future growth of the population. Yearly population size was estimated by determining the mean population size projected from 100 simulation replicates.

Sensitivity analyses were not carried out on environmental variation or catastrophes due to a lack of information with regards to how these factors affect PNP population dynamics, as well as the inability to analyse these factors in the current version of the Simulele programme. However, I note that since these analyses were undertaken the effect of a catastrophic fire on the Pilanesberg elephants has been documented and modelled (Woolley *et al*. 2008b).

Comparison of models

Absolute differences between rates of increase for the different models were calculated to determine differences between projections.

Table 1 Default parameters used for the modelling of the Pilanesberg National Park elephant population. Simulele refers to the Simulele programme that models a Leslie matrix model and a modification of Wu and Botkin's (1980) model. VORTEX refers to Lacy *et al.*'s (2003) population modelling programme.

Parameters	**Modelling programmes**	
	Simulele	**VORTEX**
Minimum age of female maturity	8 years	-
Mean age of female maturity	12 years	12 years
Mean age of maturity in males	14 years	14 years
Maximum age of reproduction	50 years	50 years
Maximum number of progeny per year	-	1
Sex ratio at birth	1 male : 1 female	50% male : 50% female
Mating system	-	Long-term polygamy
Pregnancy length	2 years	-
Anoestrous lactation length	1 year	-
Intercalving interval	3 years	33% of females can breed in any year
% Males in breeding pool	-	99
Survival probabilities for all age classes	1	0% mortality rate

Results

Observed rate of increase

Although some of the data for the observed population size estimates were missing (no data were collected), linear regression with ln transformation was conducted on several time frames to determine the observed rate of increase. The equation of the regression line for the period 1989 to 2003 was: y = 0.1143x - 223.86 (R^2 = 0.99, p < 0.0001, N = 6), with the slope of the regression (0.1143) equal to the observed mean rate of increase (r) per annum.

Modelling the 1998 to 2003 population

The observed annual rate of increase during 1998 to 2003 was 0.1065 (linear regression: R^2 = 0.98, p = 0.0953, N = 3), as calculated from the total population counts. Based on t-tests, simulations where the 'modelled' rates of increase were not significantly different from the actual observed rate of 0.107 were taken to be representative of the actual events and therefore used as information for further simulations.

The following intercalving intervals produced similar rates of increase to the observed rate: Leslie matrix model - two to four years, Wu and Botkin model - three to five years, VORTEX - two to four years (Table 2). From these results, the mean intercalving interval for further analyses was taken as three and four years, since these intervals were common to all three models. Two years of pregnancy and a minimum of one year of anoestrous lactation were simulated under these intercalving intervals. For mean age at first calving, rates of increase similar to the observed rate varied from eight to 16 years across all three models (Table 2). The mean across these years, i.e. 12 years, was used as the mean age at first calving in subsequent analyses.

Different sex ratios at birth had no effect on the rate of increase for both the Leslie and the Wu and Botkin model. For VORTEX, the simulations resulted in no more than a 0.3% difference in the rate of increase between different sex ratios (Appendix 3a). Therefore the sex ratio at birth was set at 50% female : 50% male for subsequent analyses.

No natural deaths were reported during 1998 to 2003, but seven adult bulls were hunted during this period (van Niekerk *et al.* 2001; Burke *et al.* 2008). Therefore, only adult mortality rates were analysed to determine the effect of this parameter on the rate of increase. The hunted bulls equate to an annual death rate of 0.9%, but t-tests indicate that annual death rates of up to 1.5% yielded rates of increase similar to that observed (Appendix 3b).

Table 2 Combined sensitivity analyses for the age at which a female gives birth to her first calf and the mean intercalving interval, for the period 1998 to 2003. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the observed rate of increase for 1998 to 2003 ($r = 0.107$) are given in bold (based on t-tests comparing two slopes (Zar 1996).

Mean age at first calving	Simulated rates of increase for 1998 - 2003[#]			
	Mean intercalving interval			
	2 years	3 years	4 years	5 years
		***Leslie**		
8	**0.154**	0.115	0.092	**0.077**
10	**0.147**	0.109	0.087	**0.072**
12	**0.139**	0.103	**0.081**	**0.068**
14	0.130	0.095	**0.076**	**0.063**
16	0.121	0.089	**0.070**	**0.058**
		***Wu & Botkin**		
8	**0.191**	0.114	0.095	0.079
10	**0.176**	0.108	0.087	0.073
12	**0.169**	0.105	0.084	0.071
14	**0.167**	0.104	0.083	0.070
16	**0.164**	0.103	0.082	0.069
		***VORTEX**		
8	**0.155**	0.114	0.091	**0.078**
10	**0.146**	0.108	0.086	**0.072**
12	**0.140**	0.100	0.082	**0.068**
14	0.133	0.096	**0.078**	**0.063**
16	0.129	0.092	**0.074**	**0.061**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

***Leslie** refers to the Leslie matrix model calculated using the Simulele modelling programme. **Wu & Botkin** refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. **VORTEX** refers to Lacy *et al.*'s (2003) population modelling programme.

Modelling the 1989 to 1998 population

The modified observed rate of increase per annum during 1989 to 1998 was 0.0381 (linear regression: $R^2 = 0.97$, $p = 0.0159$, N = 4). Based on t-tests, simulations where the 'modelled' rates of increase were not significantly different from the observed rate of 0.038 were taken to be representative of the actual events and therefore used as information for further simulations.

The following intercalving intervals produced similar rates of increase to the observed rate: Leslie matrix model - four to seven years, Wu and Botkin model- five to seven years, VORTEX- three to seven years (Table 3). From these results, the mean intercalving interval for further analyses was taken to be between five and seven years, since these intervals were common to all three models. For mean age at first calving, rates of increase similar to the observed rate varied from eight to 16 years across all three models (Table 3). For further analyses, 10 to 14 years was used as the age at first calving since these ages were common to all three models.

Mortality rates were determined for adult (> 15 years) and sub-adult (11 to 15 years old) bulls, since deaths in both these age groups took place during 1989 to 1998, due to either natural causes or culling (R. Slotow, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication). The deaths equate to an annual death rate of 0.7% for adult bulls and 0.6% for sub-adult males. A combined adult and sub-adult annual mortality rate of up to 2.5% yielded rates of increase similar to that observed (Appendices 4a-c).

Future Population Growth

The population increased more than five times in size over the 20-year simulation period. The projected 2023 population sizes, with the age of first calving at 12 years and a mean intercalving interval of three years, were: Leslie matrix model - 862 individuals (= 8.5% population growth rate); Wu and Botkin model - 855 individuals (= 8.5% population growth rate); VORTEX - 825 individuals (= 8.1% population growth rate). Of the reproductive parameters, mean intercalving interval had the greatest effect on the rate of increase. A one year difference in intercalving interval resulted in a 15% decrease in rate of increase, on average, compared to a two year increase in age at first calving with, on average, a 4% decrease in rate of increase (Table 4).

As the death rate increased, across all age groups, so predictably the population size decreased. Increasing the death rate in the adult age group had the greatest effect on population size (Table 5). Varying inbreeding depression, from 0 to 5.62 lethal equivalents (based on Ralls *et al*. 1988; Lacy *et al*. 2003), had no effect on the rate of increase (results not shown).

Varying the number of herd animals removed did not have a large effect on the population size. But, increasing the frequency of removals over a twenty year period resulted in a substantial decrease in the population size (Table 6). As the number of males harvested per year increased so the population decreased, with removals of more than 14 males per year leading to a negative rate of increase and population extinction (Table 7).

Table 3 Combined sensitivity analyses for the age at which a female gives birth to her first calf and the mean intercalving interval, for the period 1989 to 1998. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1989 to 1998 ($r = 0.038$) are in bold (based on t-tests comparing two slopes (Zar 1996).

Mean age at first calving	Simulated rates of increase 1989 - 1998[#]					
	Mean intercalving interval					
	2 years	3 years	4 years	5 years	6 years	7 years
			*Leslie			
8	**0.115**	**0.088**	**0.072**	**0.061**	**0.052**	**0.046**
10	**0.102**	**0.077**	**0.062**	**0.052**	0.045	0.039
12	**0.091**	**0.068**	**0.055**	0.046	0.039	0.035
14	**0.082**	**0.061**	**0.049**	0.039	0.035	**0.030**
16	**0.075**	**0.054**	0.043	0.036	**0.030**	**0.026**
			*Wu & Botkin			
8	**0.172**	**0.090**	**0.076**	**0.063**	**0.053**	0.045
10	**0.155**	**0.079**	**0.066**	**0.054**	0.046	0.039
12	**0.145**	**0.073**	**0.061**	**0.049**	0.041	0.036
14	**0.139**	**0.068**	**0.058**	0.046	0.039	0.034
16	**0.135**	**0.067**	**0.055**	0.045	0.038	0.033
			*VORTEX			
8	**0.115**	**0.088**	**0.071**	**0.060**	**0.051**	0.045
10	**0.101**	**0.075**	**0.062**	**0.051**	0.044	0.040
12	**0.088**	**0.066**	**0.053**	0.044	0.035	0.032
14	**0.077**	**0.055**	0.044	0.036	**0.030**	**0.026**
16	**0.061**	0.044	0.035	**0.027**	**0.024**	**0.020**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

* Model names as given in Table 2.

Table 4 Combined sensitivity analyses for the age at which a female gives birth to her first calf and the mean intercalving interval. Annual rates of increase (*r*) as projected by the population models are shown for the period 2003 to 2023.

Mean intercalving interval	Mean age at first calving	Projected rates of increase for 2003 - 2023[#]		
		*Leslie	*Wu & Botkin	*VORTEX
3 years	12	0.081	0.082	0.078
	14	0.077	0.079	0.073
	16	0.074	0.076	0.069
4 years	12	0.069	0.070	0.066
	14	0.065	0.067	0.062
	16	0.062	0.066	0.059
5 years	12	0.054	0.057	0.058
	14	0.057	0.059	0.054
	16	0.054	0.057	0.052

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

* Model names as given in Table 2.

Table 5 Sensitivity analyses of death rates for various age groups. Annual rates of increase (*r*) as projected by the population models are shown for the period 2003 to 2023.

Age group	% Death rate	Projected rates of increase for 2003 - 2023[#]		
		*Leslie	*Wu & Botkin	*VORTEX
Adult (> 15 years)	1	0.077	0.077	0.073
	2	0.073	0.073	0.068
	3	0.069	0.069	0.063
	4	0.065	0.065	0.058
	5	0.061	0.061	0.054
Sub-adult (6-15 years)	1	0.078	0.079	0.076
	2	0.075	0.076	0.073
	3	0.072	0.072	0.071
	4	0.069	0.070	0.069
	5	0.066	0.066	0.066
Juvenile (2-6 years)	1	0.080	0.080	0.076
	2	0.078	0.078	0.074
	3	0.076	0.077	0.073
	4	0.074	0.075	0.071
	5	0.072	0.074	0.070
Infant (< 2 years)	1	0.082	0.081	0.076
	2	0.079	0.080	0.074
	3	0.078	0.079	0.072
	4	0.078	0.078	0.071
	5	0.077	0.077	0.069

[#]Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*Model names as given in Table 2.

Table 6 Sensitivity analyses of the removal of herds of various sizes at various frequencies. Annual rates of increase (*r*) as projected by VORTEX are shown for the period 2003 to 2023.

Herd size	Projected rates of increase 2003 - 2023[#]				
	Frequency of harvesting				
	Once	Every 10 years	Every 5 years	Every 2 years	Every year
6	0.077	0.076	0.074	0.068	0.056
8	0.076	0.074	0.071	0.060	0.034
10	0.076	0.074	0.069	0.054	0.014

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*Herd composition: females: 2 x one year old, 1 x five year old, 2 x adult; male: 1 x five year old.

[†]Herd composition: females: 1 x three year old, 1 x six year old, 1 x seven year old, 1 x eight year old, 3 x adult; male: 1 x nine year old.

[‡]Herd composition: females: 2 x two year old, 1 x five year old, 1 x seven year old, 1 x nine year old, 4 x adults; male: 1 x nine year old.

Table 7 Sensitivity analyses of the removal of various numbers of males per year. Annual rates of increase (*r*) and probability of extinction of populations as projected by VORTEX are shown for the period 2003 to 2023.

Number of males harvested per year	Projected rates of increase[#]	Probability of extinction (%)
2	0.076	0
4	0.074	0
6	0.050	0
8	0.032	0
10	0.019	1
12	0.003	29
14	-0.006	78
16	-0.008	94
18	-0.011	99
20	extinct	100

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

Comparison of models

Calving intervals and age at first calving, for the 1998 to 2003 population (Table 2), showed that rates of increase were lowest for both the Leslie matrix model (50% of rates) and VORTEX (50% of rates). An absolute difference of up to 4.3% was found for rates of increase between models for a mean intercalving interval of two years. For other intercalving intervals (excluding age at first calving of 16 years) models differed by no more than 0.9%. With the age at first calving at 16 years, rates of increase between the models differed between 1.1% and 4.3%. Varying sex ratios at birth resulted in rates of increase that did not differ by more than 0.5% between models (Appendix 3a). The Leslie matrix model showed the lowest rate of increase for adult death rates (Appendix 3b). An adult death rate of 5% resulted in differences of up to 2% between models; lower death rates had differences of up to 0.8% between the models.

Comparisons of all the analyses, for the 1989 to 1998 population, showed that the majority (89%) of rates of increase were lowest for VORTEX. An absolute difference of between 5.4% and 7.5% was found for rates of increase between models when the mean intercalving interval was two years (Table 3). Other intercalving intervals (excluding age at first calving of 14 and 16 years), had rates of increase differing by no more than 0.8% between the models. With age at first calving of 14 and 16 years, rates of increase between models differed between 0.9% and 7.5%. With varying mortality rates, rates of increase between models did not differ by more than 1.6%, with 82% of the analyses differing by less than 0.9% (Appendices 4a-c).

For future projections, VORTEX showed the lowest rates of increase. Projections of various intercalving intervals and ages at first calving resulted in rates of increase that differed by no more than 0.8% between the models (Table 4). Sensitivity analyses of various death rates for various age groups resulted in rates of increase that did not differ by more than 0.7% between models (Table 5). The Leslie matrix model and the modified Wu and Botkin model gave similar rates of increase, in comparison to VORTEX.

Discussion

PNP's population growth rate of 12.1% is well above the maximum growth rate of 7% as predicted by Calef (1988), and much higher than many of the 'natural' populations of elephants in South Africa, i.e. 5.8% for the AENP population (Gough & Kerley 2006) and 6.6% for the KNP population (Whyte 2001). The PNP growth rate is, however, well within the range for small, fenced-in populations of elephants in South Africa, which may be as high as 16% (van Jaarsveld *et al*. 1999; Garaï *et al.* 2004; Slotow *et al.* 2005; Mackey *et al*. 2006). High growth rates have been observed in elephant populations in eastern and southern Africa, which are recovering from poaching (Jachmann 1986; Blanc *et al*. 2005; Ogola & Omondi 2005; Junker *et al*. 2008). Many of the small populations of elephants in South Africa do not follow Calef's (1988) assumptions. For example, the PNP population initially consisted of a female-skewed sex ratio with most individuals being juveniles. By 1998 the population continued to have a female-skewed sex ratio, with 37.2% of the total population comprising females above 12 years of age (mean age of first calving). These conditions are not unique to this population and were observed in many other small populations of elephants (Garaï *et al.* 2004; Slotow *et al.* 2005; Mackey *et al*. 2006), as well as the post-poaching population of Tsavo East National Park (Kenya, McKnight 2000). Studies have similarly attributed the high growth rate of these populations to female-biased adult sex ratios, cohorts of similarly aged individuals that reach sexual maturity at the same time, an extremely low mortality rate, a lower age of first reproduction as well as a lower mean intercalving interval (Garaï *et al.* 2004; Slotow *et al.* 2005; Mackey *et al*. 2006). A high growth rate is found when interrogating the initial growth of large stable elephant populations in South Africa. Prior to the complete fencing of the KNP in 1976, the elephant population had an average annual growth rate of 10% (van Jaarsveld *et al*. 1999; Whyte 2001). Although much of this growth has been attributed to immigration and emigration from neighbouring populations in Zimbabwe and Mozambique (Whyte 2001), one can consider that the area of the KNP was favourable for elephant population growth. Similarly a maximal growth rate of 11% was found for the Amboseli elephant population, in Kenya, whilst the population was recovering from a population crash due to poaching and drought (Moss 2001). Growth rates of up to 11.2% have been found for elephants in northern Botswana between 1973 and 1993 (Junker *et al*. 2008). Thus all these populations could be considered young, growing (potentially eruptive (Caughley, 1970) populations.

The PNP population prior to the introduction of adult males (i.e. 1989-1998) had an average annual population growth rate of 3.9 %, a mean intercalving interval of between five and seven years, a mean age at first calving of between 10 and 14 years and a mean annual death rate of less than 2.5%. In comparison, the 1998-2003 period had an average annual population growth rate of 11.2%, a mean

intercalving interval of between three and four years, a mean age at first calving of 12 years and a mean annual death rate of less than 1.5%. Many factors could be considered to be the cause of the slow growth rate of the pre-1998 population. Some of these possibilities include a hiatus due to the sexual inexperience of immature animals, insufficient numbers of sexually mature individuals (only 13.7% of the population comprised of females above 12 years of age) or the stress of translocation. The droughts experienced in 1992 and 1994 (data from South African Weather Service) could have had an effect on conception rates resulting in lower numbers of births, thus a lower growth rate. Lower birth rates were recorded in the two years after the end of a drought in Amboseli National Park (Kenya, Moss 2001). Since a decrease in rainfall is likely to have an effect on the nutritional status of food plants available, the nutritional status of the cow is depressed, and thus the onset of oestrus is delayed (Laws *et al*. 1975; Viljoen 1988). Birth peaks two years after good rainfall conditions were similarly recorded in Amboseli National Park, Samburu and Buffalo Springs National Reserves (Kenya, Moss 2001; Wittemyer 2001; Wittemyer *et al*. 2005).

The mean intercalving intervals are within the lower range of intervals of populations in southern and eastern Africa, which vary between three and seven years (see Appendix 5). Asian elephants (*Elephas maximus*) have similar intercalving intervals of between four and five years (Sukumar 2003). The intercalving interval of three years during 1998-2003 may appear surprising, since this equates to only one year of anoestrous after calving. Although this is generally rare, similar intercalving intervals were found in other small populations in South Africa where the intercalving interval has ranged from 2.4 to 3.9 years (Mackey *et al*. 2006). Mackey *et al*. (2006) identified an intercalving interval of 3.29 years, for the PNP population, from known intercalf intervals of females with more than one calf. The apparent decrease in intercalving interval post-1998 may be associated with a more stable social structure, i.e. with the introduction of mature males and young animals reaching sexual maturity, as well as the absence of factors, such as translocation stress, and drought conditions, that may have affected reproduction in the founding population.

The mean age at first calving is within or just under the mean age at calving of many populations in southern and eastern Africa (see Appendix 5). Asian elephants show similar variation in age at first calving, ranging from 10 to 12 years in Sri Lanka (McKay 1973; Kurt 1974; Ishwaran 1993; Katugaha *et al*. 1999) to 17 years in southern India (Sukumar 1985; Sukumar 1989). Mackey *et al*. (2006) found values ranging from 8.3 to 12.3 years within small populations in South Africa, with PNP females maturing at 9.18 years of age, thus age at first calving would be approximately 11 years. Mackey *et al*. (2006) calculated age at maturity from "all known mother-calf relationships with first-born elephants of known age", whereas in this chapter age at first calving was determined by finding ages which

resulted in rates of increase not significantly different to the actual rate of increase. Mean age at first calving is likely to be influenced by the same factors that influence intercalving interval. The post-1998 population appears to have matured much earlier than the founding individuals, possibly due to more favourable conditions during this period as well as the presence of sexually mature adult bulls. This decrease in the age at maturity has been found in the Murchinson Falls National Park population, where during 1966 to 1967 age at maturity ranged from 16.3 years and 17.8 years for the northern and southern subpopulations respectively (Laws *et al.* 1975) and then decreased to 9.6 years and 9.0 years for the same subpopulations in 1974 (Malpas 1978; Sukumar 2003). Intercalving interval thus appears to have the greatest effect on the PNP's rate of increase. This conclusion agrees with studies by Hanks and McIntosh (1973), Whyte (2001) and Mackey *et al*. (2006).

Large disparities between the observed mean annual death rate and the annual death rate that approximated the actual population growth were found for both time periods modelled. One possible explanation is the inexperience of the young females with regards to calf care, as well as the drought conditions of 1992, 1994, and 1999 may have resulted in many young calves having died without being noticed by staff at PNP. The carcasses of young calves are quickly disposed by predators, leaving no visible remains (McKnight 2000; Whitehouse 2001; Moss 2001). Another reason for the disparity in death rates may be due to several assumptions made by the models, since models are a simplification of an observed process and the many intricacies of reality cannot be translated into a model (Jeffers 1982; Starfield & Bleloch 1991). The lack of detailed information on the population and the many assumptions regarding the age of reintroduced individuals may have resulted in rates of increase similar to actual rate, but not necessarily representative of actual events.

Growths rates of between 8.1% and 8.5%, for 2003-2023, were above Calef's (1988) maximum population growth rate, but lower than over the period 1989-2003. Clearly, if conditions stay similar to the 2003 conditions (including no deaths), the population is expected to lower its population growth, in the short-term (20 years), since the population over the course of the simulation will begin to stabilise. By the end of the simulation period approximately 24% of the population comprised females above 12 years of age, compared to 37.2% in 1998 and 38.2% in 2003. The percentage of reproductive females in a population has a large effect on a population's growth potential, since it directly determines the number of calves produced (Calef 1988; Garaï *et al*. 2004; Slotow *et al*. 2005; Mackey *et al*. 2006). Examples of this in other elephant populations include: an estimated 7% annual recruitment rate for 1971-1972 in the Gonarezhou National Park population where 33% of the population comprised mature females (Sherry 1975), a recruitment rate of 9.8% calculated for the KNP population when 39.6% of the population comprised mature females (Whyte 2001) and the

Mabula Game Reserve which showed an annual growth rate of 15.3% with mature females comprising 40% of the total population (Mackey *et al*. 2006). As the population age structure stabilises, so the percentage of reproductive females in a population will decrease, producing fewer calves, leading to a decrease in growth rate (Kerr 1978; Mackey *et al*. 2006). The sex ratio over the course of future simulations approached a 1 : 1 ratio, with 49% to 53% of the population consisting of females, in comparison to the 2003 population where 57% of the population consisted of females.

Inbreeding depression is not likely to have an effect on an elephant population over 20 years of simulations. This can be attributed to the elephant's life history traits, most noteworthy of which is the fact that elephants live for approximately 60 to 65 years (Moss 2001). Clearly, the effects of inbreeding will not be visible in a single human life-time in a species with a long life-span, late age of sexual maturity, long intercalving interval, long generation time and a polygynous mating system where males act as migrants between female herds (Sukumar 1989; Mills & Hes 1997; Skinner & Chimimba 2005). Whitehouse (2001) did not find any noticeable signs of inbreeding depression in the AENP population where fecundity is high and mortality is low despite the population having gone through a severe population bottleneck in 1931. Despite this, attempts to prevent inbreeding should be taken into consideration since inbreeding depression has now been observed in other species in the wild (Keller 1998; Crnokrak & Roff 1999; Eldridge *et al*. 1999; Hedrick & Kalinowski 2000; Frankham *et al*. 2002; Keller & Waller 2002; Frankham 2005). Simulations of elephant populations have shown increased extinction risk due to inbreeding depression after 25 or more generations (Brook *et al*. 2002b, Reed *et al*. 2003, Reed 2004).

Of the demographic factors, adult death had the greatest effect on the population growth. These results are in concordance with the studies by Dominy *et al*. (1998) and Mackey *et al*. (2006). Dominy *et al*. (1998) stated that the effect of increasing mortality in the adult age group has a large effect on population growth not only due to a reduction in the number of animals but due to a decrease in number of breeding individuals. But, these results are in contrast to Fowler and Smith's (1973) and Hanks and McIntosh's (1973) model projections where infant mortality (calves < 2 years old) had the greatest effect on the rate of increase. These populations may have had sufficient numbers of adult animals to reproduce, however the future reproductive potential of the population was drastically reduced by the death of infants.

The annual removal of males had a much greater effect on the population growth than the removal of an equal number of herd individuals. A decrease in the number of sexually mature males leads to a decrease in reproduction resulting in a decrease in population growth. In AENP, the population

experienced a decrease in population growth between 1938 and 1948 after the removal of the only two sexually mature bulls from the population (Whitehouse 2001). Ogola and Omondi (2005) similarly expect the Sweetwaters Game Reserve population to have a low growth rate due to the adult population being highly skewed towards females after the translocation of 57 individuals to Meru National Park in 2001. The annual removal of adult males is a viable short-term management option. Over and above reducing population size over the short-term, the removal of males can be used as a source of income when these animals are hunted (Slotow *et al*. 2008). But, this option is not without its complications. The absence of mature adult males from a population is known to cause abnormal behaviour in adolescent males (Slotow *et al*. 2000; Slotow & van Dyk 2001), and the removal of individuals may have an effect on the genetic diversity of a population (see Chapter 4). Thus if adult male removal is considered as an option, careful decisions will have to be made with regards to which individuals should be removed.

Comparisons of rates of increase obtained from projections by the different models do not show very large differences. Adult death rates of 5% in the 1989 and 1998 populations resulted in the greatest differences between models when varying death rates. This may be due to the different model assumptions as well as the way death rates were simulated. VORTEX simulates death rates for either male or female or both sexes, whereas in the Simulele programme only death rates for both sexes can be simulated. The fewest number of differences were seen between models for the period 2003-2023. The time period of 20 years may have resulted in lower variations in population growth, compared to the shorter time periods of five years for the 1998 population and nine years for the 1989 population. The closer similarities between the Leslie matrix model and the Wu and Botkin model for the future population may be due to the same input interface used by both models, resulting in differences to VORTEX results. Differences between the models may be due to the various factors considered and the way they are simulated. The Leslie matrix model is a deterministic model and thus will result in rates of increase differing from the stochastic Wu and Botkin and VORTEX models. But, the input data required by the Wu and Botkin model and VORTEX are not identical, and thus variations in simulations of the same situation may occur resulting in different rates of increase. VORTEX in general appears to produce the lowest rates of increase, and has also been found to produce lower rates of increase in comparison to a life-table analysis prediction for a population of whooping cranes (Lacy 1993). This result was attributed to factors such as inbreeding and imbalanced sex ratios which are not considered in deterministic life-table calculations (Lacy 1993). Despite the small differences between the deterministic Leslie matrix model and the stochastic Wu and Botkin and VORTEX models, the stochastic models are more realistic for small populations and therefore more appropriate for further analyses thereof (Sukumar 2003).

In conclusion, the PNP population has increased at rates well above which Calef (1988) predicted, but within the range of other small, fenced-in populations in South Africa (van Jaarsveld *et al*. 1999; Garaï *et al.* 2004; Slotow *et al.* 2005; Mackey *et al*. 2006). Modelling of the historical population shows that demographic parameters of intercalving interval and age at first calving are in the lower range of values in comparison to other populations in eastern and southern Africa, which would result in the high population growth rates. Future projections predict that the population will still continue to grow at rates above 7% per annum, but below those experienced historically. Sensitivity analyses indicated that intercalving interval is the most sensitive of demographic factors, but controlling this factor in a population such as PNP via management is a near impossibility (see however a discussion on immunocontraception by Bertschinger *et al*. (2008). Sensitivity analyses of potential management strategies indicated that the removal of adult males was the most sensitive parameter. This is a realistic option, but further studies need to be conducted to determine its viability. Comparisons of the three models studied indicate that stochastic models are more representative of reality. Simulations of other elephant populations should be conducted to further compare the reliability of the Wu and Botkin model and VORTEX.

Chapter 3[2]

Non-invasive DNA sampling from elephant dung - A pilot study

Abstract

Over the last twenty years the use of non-invasive DNA sampling techniques for population genetic studies of free-ranging animals has steadily increased. Of the various sources of DNA available, faecal samples are the easiest to obtain. DNA obtained through non-invasive sampling is of both low quantity and quality, which leads to various problems. These may lead to difficulties during polymerase chain reaction (PCR) amplifications or genotyping errors resulting in insufficient or incorrect data. Due to these problems, a pilot study was conducted to critically evaluate previously described faecal storage and faecal DNA extraction methods for efficiency and repeatability under local conditions. The quality and quantity of the faecal DNA obtained was evaluated by PCR amplification of mitochondrial DNA (mtDNA) and microsatellite loci. Results show that storage of dung in a DMSO/saturated NaCl solution provided the best DNA quality. Sufficient DNA for PCR was obtained using: (1) QIAamp Stool kit, (2) Boom *et al.* (1990), and (3) Reed *et al.* (1997) Method B extraction methods. MtDNA cytochrome *b* (cyt *b*) amplifications consistently produced 400 bp and 800 bp PCR products. The 400 bp product aligned to elephant cyt *b* gene region. The 800 bp product partially aligned with parts of the *Ceratocystis fimbriata* (a fungal plant pathogen) genome, highlighting the problem of environmental contamination of non-invasive DNA sampling. Amplifications of the same samples three months after initial extractions were unsuccessful. Attempts to amplify DNA using elephant-specific D-loop primers and microsatellites were unsuccessful. Under current conditions, this study showed that obtaining sufficient quantities and quality of DNA from elephant dung was not possible.

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Introduction

Over the last twenty years, an increasing number of molecular population genetic studies of free-ranging animal populations have reported the use of non-invasive DNA sampling techniques (see amongst others: Kohn & Wayne 1997; Taberlet *et al.* 1999; Beja-Pereira *et al*. 2009). These techniques have included obtaining DNA from shed hairs (Taberlet & Bouvet 1992; Taberlet *et al.* 1997; Marshall & Ritland 2002; Clifford *et al*. 2004; Roon *et al*. 2005), shed feathers (Segelbacher 2002; Petersen *et al*. 2003; Rudnick *et al*. 2005), urine (Yokota *et al.* 1998; Valiere & Taberlet 2000), and faeces (Kohn *et al.* 1995; Reed *et al.* 1997; Kohn *et al.* 1999; Okello *et al*. 2005; Gobush *et al*. 2009). Of these techniques, faecal samples are relatively easy to obtain in the wild (Fernando *et al*. 2003; Beja-Pereira *et al*. 2009).

It is possible to obtain DNA from faeces since intestinal epithelial cells are shed during defecation (Kohn & Wayne 1997). Specific DNA sequences can then be amplified from these epithelial cells using the polymerase chain reaction (PCR, Saiki *et al*. 1985) (Kohn & Wayne 1997; Hofreiter *et al*. 2001; Morin *et al*. 2001). Even minute amounts of DNA can be amplified in this way. DNA obtained from faeces can be used to assess genetic structure, demography, life history, population subdivision, food habits, reproduction and sex ratios (Kohn & Wayne 1997; Reed *et al.* 1997; Taberlet *et al*. 1997; Kohn *et al*. 1999; Ernest *et al*. 2000; Farrel *et al*. 2000; Vidya *et al*. 2005a; Piggott *et al* 2006; Gobush *et al*. 2009). This information is of use in the field of conservation biology, where many unknown aspects of a species' biology may be addressed, especially for elusive species. These methods are particularly useful where capturing individuals is undesirable, such as in behavioural studies or in small endangered populations (Tikel *et al*. 1996; Kohn & Wayne 1997; Taberlet *et al.* 1999; Ernest *et al*. 2000 O'Ryan *et al*. 2001; Wehausen *et al*. 2004; Piggott *et al*. 2006; Eggert *et al*. 2008).

DNA obtained through non-invasive sampling is of both low quantity and quality which leads to various problems. These include the possibility of DNA contamination from other sources of DNA, the poor amplification of DNA samples due to the presence of PCR inhibitors, the possibility of genotyping errors due to 'allelic dropout' or due to the generation of 'false alleles' and DNA degradation (Taberlet *et al*. 1996; Kohn & Wayne 1997; Taberlet *et al*. 1997; Goosens *et al*. 1998; Taberlet *et al.* 1999; Bradley & Vigilant 2002; Murphy *et al*. 2002; Creel *et al*. 2003; Fernando *et al*. 2003; Murphy *et al*. 2003; Eggert *et al*. 2005; Broquet *et al*. 2007; Beja-Pereira *et al*. 2009).

The epithelial cells shed from the intestinal lining (Kohn & Wayne 1997) are mostly found on the outside of the faeces, exposing the DNA from these cells to decay because of the environment in

which the faeces are found. Since the intestinal cells present on the faeces are 'dead' the repair mechanisms found in living cells are not present; the DNA is exposed to both hydrolytic and oxidative decomposition and is spontaneously degraded at moderate temperatures, such as those that would be present in the field (Lindahl 1993). An environment with continued ambient moisture, exposure to sunlight and continual bacterial degradation will result in DNA degraded to such an extent that it can no longer be used in PCR amplification (Foran *et al*. 1997; Reed *et al*. 1997; Hofreiter *et al.* 2001; Fernando *et al*. 2003).

Due to the environmental effects on DNA, the storage of dung samples is thus extremely important (Taberlet *et al*. 1999, Beja-Pereira *et al*. 2009). Previous studies have compared the effectiveness of various storage methods on the success of obtaining amplifiable DNA (Wasser *et al*. 1997; Frantzen *et al*. 1998; Murphy *et al*. 2002; Fernando *et al*. 2003). Dung can be stored dry (for example: air dried, silica dried) or in a liquid storage medium (for example: DMSO/saturated NaCl solution, ethanol). Although the storage of elephant dung in DMSO/saturated NaCl solution has been used successfully in other elephant studies (Nyakaana & Arctander 1999; Eggert *et al*. 2002; Nyakaana *et al*. 2002; Fernando *et al*. 2003; Okello *et al*. 2005; Eggert *et al*. 2008; Gobush *et al*. 2009), one should heed Frantzen *et al*.'s (1998) advice that "results will possibly vary according to ... ecological conditions at the study site".

DNA that is present in numerous copies within a single cell is more likely to survive DNA decay (Hofreiter *et al.* 2001). For example, animal mitochondrial DNA (mtDNA) is present in multiple copies in a single cell. Therefore, it is possible to obtain good quality mtDNA from faecal samples for further analysis (Kohn & Wayne 1997; Hofreiter *et al.* 2001). On the other hand, single copy nuclear DNA, such as microsatellites, is degraded into small fragments and is present in very low quantities after environmental degradation (Taberlet *et al.* 1996; Taberlet *et al.* 1999). As a result: (1) PCR amplification may not be possible, (2) a PCR product with the correct genotype may be obtained, or (3) a PCR product with the incorrect genotype may be obtained (Taberlet *et al.* 1999).

Because PCR can amplify minute quantities of DNA, the risk of contamination of faecal DNA extracts with non-target DNA is large (Kohn & Wayne 1997; Taberlet *et al.* 1999). To overcome this problem several guidelines are generally followed: the physical isolation of areas for DNA extraction and PCR preparation, the use of disposable gloves, the use of aerosol resistant filter pipette tips as well as the use of positive and negative controls during DNA extraction and PCR preparation (Kwok & Higuchi 1989; Taberlet *et al.* 1996; Reed *et al.* 1997; Wasser *et al.* 1997; Kohn *et al.* 1999; Ernest *et al.* 2000; Segelbacher 2002; Eggert *et al*. 2005; Beja-Pereira *et al*. 2009).

Amplification rates of non-invasive DNA can be further reduced by the presence of PCR inhibitors co-extracted with the DNA. These PCR inhibitors may be of plant origin from the animal's diet (O'Ryan *et al.* 2001), may be due to bilirubin and bile salts (Deuter *et al.* 1995) or from other unknown inhibitors (Pääbo 1990). To overcome inhibition, various chemicals such as bovine serum albumin (BSA, Pääbo 1990), hexadecyltrimehylamonium bromide (CTAB, Constable *et al.* 1995), or polyvinylpyrrolidone (PVP, O'Ryan *et al.* 2001) are used.

As a result, the DNA extraction technique is a crucial step in any non-invasive genotyping study. The ideal DNA sample would be one of high quality and quantity, with the absence of any PCR inhibitors. There are no standard protocols for DNA extraction from faeces (Eggert *et al*. 2005); including elephant dung (see Eggert *et al*. 2002; Nyakaana *et al*. 2002; Fernando *et al*. 2003; Okello *et al*. 2005; Gobush *et al*. 2009). Newer DNA extraction techniques which include a purification step, such as Boom *et al*.'s (1990) method where DNA is adsorbed onto silica beads, have shown much promise in faecal DNA studies (Eggert *et al.* 2005; Beja-Pereira *et al*. 2009).

Previous African elephant (*Loxodonta africana*) genetic studies have obtained DNA from tissue samples of culled animals (Osterhoff *et al*. 1974; Georgiadis *et al*. 1994; Coetzee *et al*. 1999; Whitehouse & Harley 2001), tissue samples obtained by biopsy darting (Karesh *et al*. 1987; Georgiadis *et al*. 1994; Nyakaana & Arctander 1999; Nyakaana *et al*. 2001; Roca *et al*. 2001; Whitehouse & Harley 2001; Comstock *et al*. 2002; Nyakaana *et al*. 2002) or tissue/blood samples from animals immobilised for veterinary attention or for translocation (Osterhoff *et al*. 1974; Georgiadis *et al*. 1994). Behavioural studies of elephant populations in small game reserves in South Africa (Burke *et al.* 2008) make the use of conventional methods for obtaining DNA samples, such as biopsy darting (Karesh *et al*. 1987), undesirable. More recently, studies have used dung as a source of DNA (Nyakaana & Arctander 1999; Nyakaana *et al*. 2002; Eggert *et al*. 2002; Okello *et al*. 2005; Eggert *et al*. 2008; Archie *et al.* 2008; Okello *et al*. 2008a, b; Gobush *et al*. 2009), with Okello *et al*. (2005) reporting up to a 98% success rate in correctly genotyping 202 individual elephants. The ease with which dung samples can be obtained and the possibility of sampling large numbers of individuals in a population make non-invasive sampling a potentially valuable technique to investigate for further studies.

Before a large-scale molecular study using faecal DNA can be carried out, a pilot study to determine the feasibility of faecal DNA for a population genetic study is critical (Taberlet *et al*. 1999; Beja-Pereira *et al*. 2009). The aim of this chapter was to determine whether elephant dung could be a reliable source of DNA for a population genetic study of South African elephants. To determine the

feasibility of using faecal DNA, previously described methods for faecal storage and faecal DNA extraction, of which many are previously described for non-herbivores, were critically evaluated for efficiency and repeatability under local conditions. Furthermore, the quality and quantity of faecal DNA obtained was evaluated by PCR amplification of mtDNA and microsatellite loci.

Materials and methods

Sample Collection

Fresh elephant dung was obtained by Zoo staff from the two resident female elephants at the National Zoological Gardens, Pretoria, South Africa from the 5th to the 7th of March 2001 and transported to the Department of Genetics, University of Pretoria on the 7th of March (see Appendix 6 for the relevant permit). The average temperature over the three sampling days was a minimum of 18.9°C and maximum of 31.3°C, with no rainfall (data from South African Weather Service). The wild caught elephants, originally from the Kruger National Park, were estimated to be 17 (Landa) and 16 (Pumbi) years old at the time. Their diet consisted of mixed vegetables (beetroot, pumpkin, potatoes and carrots) and *Eragrostis* hay (A. Tordiffe, National Zoological Gardens, Pretoria, personal communication).

Prior to storage, samples were thoroughly mixed. Dung samples were stored according to various methods, namely:

(a) in 20% DMSO/saturated NaCl solution containing EDTA (Amos & Hoelzel 1991);
(b) in 20% DMSO/saturated NaCl solution not containing EDTA (Amos & Hoelzel 1991);
(c) in 70% ethanol;
(d) in 100% ethanol;
(e) at -20°C;
(f) at room temperature;
(g) with silica beads (Wasser *et al*. 1997).

DNA extraction

Total genomic DNA was extracted from each storage method using the following extraction procedures:

(a) Commercially available DNA extraction kit - QIAamp Stool Kit (Qiagen);
(b) Guanidium thiocyante (GuSCN) - silica DNA purification as described by Boom *et al.* (1990);
(c) DNA isolation using 6% Chelex - 100 resin (BioRad) as described by Paxinos *et al.* (1997);
(d) DNA isolation using 6% Chelex - 100 resin (BioRad) as described by Reed *et al.* (1997) - (Method B), followed by standard phenol/chloroform extraction (Sambrook *et al*. 1989);
(e) Reed *et al.* (1997), using Chelex - 100 resin (BioRad) followed by standard phenol/chloroform extraction (Sambrook *et al.* 1989) without Proteinase K and RNase A;
(f) Boiling of samples in phosphate - buffered saline and water for 5 min as described by Saiki (1990);
(g) Boiling of samples in lysis buffer for 15 min as described by Valsechi (1998);
(h) Valsechi (1998) extraction followed by standard phenol/chloroform extraction (Sambrook *et al.* 1989);
(i) Valsechi (1998) extraction followed by standard phenol/chloroform extraction (Sambrook *et al.* 1989) without Proteinase K;
(j) Boiling of samples in water as described by Reed *et al.* (1997) - (Method A);
(k) Reed *et al.* (1997) - (Method A) followed by standard protocol described in (l) below;
(l) Standard protocol of chemical digestion (50 mM Tris pH7.6, 100 mM NaCl, 1 mM NaEDTA pH 8.0, 10% SDS, 10 mg/ml Proteinase K, 0.1 mg/ml RNase A) followed by standard phenol/chloroform extraction and ethanol/sodium acetate precipitation (Sambrook *et al.* 1989);
(m) SDS/proteinase K digestion as described in Flagstad *et al.* (1999) followed by standard phenol/chloroform extraction (Sambrook *et al.* 1989) instead of a Dynabeads extraction (Rudi *et al.* 1997);
(n) Digestion in lysis buffer containing CTAB, incubation at 60°C for one hour followed by extraction with chloroform - isoamyl alcohol as described by Launhardt *et al.* (1998).

To monitor for possible contamination during extraction, extraction blanks (containing no dung extract) were included with each extraction protocol. The quantification of DNA via fluorometry was not conducted since faecal pigments, plant DNA and bacterial DNA coextracted with the faecal DNA would result in incorrect readings (Morin *et al*. 2001). The success of the DNA extractions was determined by visual comparison of 5µl of extracted DNA to the known concentration of 5 µl of 100

bp DNA ladder (Promega), which were electrophoresed concurrently through 1% agarose gels (Laboratory Specialist Services).

PCR amplification

To determine whether DNA extracted from elephant dung could be used for further molecular analysis, amplification of mtDNA as well as microsatellite loci was carried out.

A 400 bp fragment of the mtDNA cytochrome *b* (cyt *b*) gene was amplified using universal mammal cyt *b* primers, L14841 (5'- CCA ACA TCT CAG CAT GAT GAA A- 3') and H15149 (5'- CCC TCA GAA TGA TAT TTG TCC TCA- 3') (Kocher *et al.* 1989). A 400 bp fragment of the 5' end of the control region was amplified using primers LafCR1 (5'- GTA TAA GAC ATT ACA ATG GTC- 3') located in the $tRNA^{Pro}$ gene, and LafCR2 (5'- AGA TGT CTT ATT TAA GAG GA- 3') located in the first conserved sequence block of the control region (Nyakaana & Arctander 1999), developed specifically to amplify this region of mtDNA in the African elephant. Amplifications were carried out in 50 µl reaction volumes containing 2 µl of extracted DNA (unknown concentration), 2.5 mM $MgCl_2$, 1 x buffer, 50 pmol of each primer, 1 unit of Super-Therm *Taq* DNA polymerase (Southern Cross Biotechnology) and 2.0 mM dNTPs (Promega). The PCR amplification protocol was: 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 50˚C for 30 s, 72˚C for 30 s and final extension at 72˚C for 3 min. PCR products were electrophoresed through 1% agarose gels to determine whether amplification was successful. Amplifications that showed clear bands were sequenced to determine the products that were amplified. Each band was cut out of the agarose gel and purified using the High Pure PCR Product Purification kit (Boehringer Mannheim) according to the manufacturer's protocol. Purified PCR products were sequenced by the dideoxy chain-termination method (Sanger *et al.* 1977), by amplifying 2 µl of PCR products in a final volume of 10 µl with 3.2 pmol of primer H15149 and 2 µl Big Dye ready reaction mix (Applied Biosystems). The standard cycle sequencing protocol (25 cycles of: 96˚C for 10 s, 50˚C for 5 s and 60˚C for 4 min) for amplification of PCR products was followed (Perkin Elmer 1995). Products were precipitated according to the standard sodium acetate precipitation protocol as described by Perkin Elmer (1995). The PCR products were sequenced on an ABI 377 automated sequencer (Applied Biosystems).

Microsatellite loci LafMS02 (Nyakaana & Arctander 1998) and LA4 (Eggert *et al.* 2000) isolated from the African elephant genome were used to amplify nuclear DNA. Amplifications were carried out in 10µl reaction volumes containing 2 µl of extracted DNA, 1.5 mM $MgCl_2$, 1 x buffer, 10 pmol of each primer, 0.5 units of Super-Therm *Taq* DNA polymerase (Southern Cross Biotechnology), 0.2 mM dNTPs (Promega) and 1 µl of Betaine (Sigma). Amplification conditions were: 94˚C for 3 min;

40 cycles of 94˚C for 30 s, 56˚C for 1 min, 72˚C for 1 min and final extension at 72˚C for 5 min. PCR products were electrophoresed through 2% agarose gels to determine whether amplification was successful.

All PCR amplifications included extraction blanks, as well as negative (reagent only) and positive (known DNA) controls to monitor contamination. If any of these controls were found to show contamination, the PCR results were discarded.

Literature review

Articles related to faecal DNA studies published in *Molecular Ecology* (1992-2009) and *Conservation Genetics* (2000-2009) were reviewed to determine the number of articles published per dietary guild (i.e. carnivores, omnivores, hindgut fermenting herbivores and ruminant herbivores). Only studies related to independent research were considered, all review articles were excluded.

Results

Sample storage and DNA extraction

Samples stored with silica beads were discarded due to mould growth and concurrent contamination. The combined results of storage methods and DNA extractions are given in Table 1. Samples stored in DMSO/saturated NaCl solution provided the best DNA quality, whereas samples stored in 70% or 100% ethanol showed the poorest DNA quality compared to other storage methods. Sufficient DNA for PCR was obtained using the QIAamp Stool kit, Boom *et al*. (1990) or Reed *et al*. (1997) Method B extraction methods, whereas, the Saiki (1990) method and the Flagstad *et al.* (1999) method with a standard phenol/chloroform extraction performed the worst over all storage methods with no DNA obtained.

PCR amplification

Mitochondrial cyt *b* amplifications were most successful with samples stored in DMSO/saturated NaCl with or without EDTA or at -20°C followed by extraction via the QIAamp Stool kit, Boom *et al.* (1990) or Reed *et al.* (1997) Method B. Amplifications of these same samples approximately 3 months after extraction were unsuccessful.

Table 1 DNA quality obtained from various sample storage methods and extraction protocols. Details of storage methods and extraction protocols are described in the 'Materials and methods' section of the text. Extracts were visualised on 1% agarose gels, and scored by comparison to a sample of known DNA concentration.

Extraction protocol	Storage method					
	DMSO/NaCl with EDTA	DMSO/NaCl without EDTA	70% Ethanol	100% Ethanol	-20°C	Room temperature
QIAamp Stool Kit	n/a	low	n/a	n/a	high	n/a
Boom (1990)	n/a	low	n/a	n/a	DNA	n/a
Paxinos (1997)	low	low	none	none	none	none
Reed (1997) method B	DNA	low	none	none	none	low
Reed (1997) method B + Phenol extraction	DNA	low	none	none	none	none
Saiki (1990)	none	none	none	none	none	none
Valsechi (1998)	low	low	none	none	none	low
Valsechi (1998) + Phenol extraction	DNA	low	none	none	none	none
Valsechi (1998) + Phenol extraction without Proteinase K	low	low	none	none	none	none
Reed (1997) method A	low	none	none	none	low	low
Reed (1997) method A + Phenol extraction	low	low	none	none	none	low
Standard Phenol/ Chloroform extraction	high	low	low	none	low	DNA
Flagstad (1999) + Phenol extraction	none	none	none	none	none	none
Launhardt (1998)	DNA	DNA	low	none	low	DNA

Key: n/a - DNA extraction not attempted, none - no DNA visible on agarose gel, low - low intensity DNA smear visible on agarose gel, DNA - clear DNA smear visible on agarose gel, high - high intensity DNA smear visible on agarose gel.

Of these successful cyt *b* amplifications two clear bands, 400 bp and 800 bp respectively, were consistently found on agarose gel electrophoresis (Fig. 1). Sequences were successfully produced from the 400 and 800 bp fragments respectively. To determine the source of the DNA amplified, a BLAST search (Altschul *et al.* 1997) was conducted on the sequences obtained from each band. The 400 bp sequence aligned to African 'forest' elephant (*Loxodonta cyclotis*) and African elephant cyt *b* gene with 99% identity. Nine sequences from the GenBank, EMBL, DDJB and PDB databases had the highest bit score of 620 with an E-value = 6e-175. Eight of these nine alignments were various haplotypes of the cyt *b* gene of African elephant (Fig. 2). The ninth alignment was a sequence from the cyt *b* gene of a forest elephant.

The 800 bp band sequence aligned to different strains of *Ceratocystis fimbriata* (a fungal plant pathogen, Witthuhn *et al*. 1999) internal transcribed spacer gene 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 with 97% identity (Witthuhn *et al.* 1999) (Fig. 3). Five sequences from the GenBank, EMBL, DDJB and PDB databases had the highest bit score of 821 with an E-value = 0. Primer sequences could not be found in the full *Ceratocystis fimbriata* internal transcribed spacer gene 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 sequence (GenBank accession number: AY233863). Alignment of the *C. fimbriata* sequence (GenBank accession number: AY233863) and the 800 bp sequence in ClustalX (Thompson *et al*. 1997) showed partial alignment with many gaps introduced into the *C. fimbriata* sequence (Fig. 4).

Amplifications with LafCR1 and LafCR2 were unsuccessful, many either showing a DNA smear, presence of primer dimers or no amplification product on agarose gels. Attempts to optimise PCR conditions by varying the $MgCl_2$ concentration and annealing temperature (Kidd & Ruano 1995; Roche Molecular Biochemicals 1999) were unsuccessful. Amplifications that showed contamination were discarded, as the source of contamination could not be determined.

Amplifications with microsatellite loci LA4 and LafMS02 were unsuccessful, either showing a DNA smear, presence of primer dimers or no amplification product on agarose gels. Further attempts to optimise PCR conditions by varying $MgCl_2$ and annealing temperatures (Kidd & Ruano 1995; Roche Molecular Biochemicals 1999) were unsuccessful.

Literature Review

A total of 122 published articles stated the use of faeces as a source of DNA for further molecular genetic studies. Of these studies only 10% (12) used faeces from hindgut-fermenting herbivores, with the majority of studies (45%) using faeces from carnivores (Fig. 5, Appendix 7).

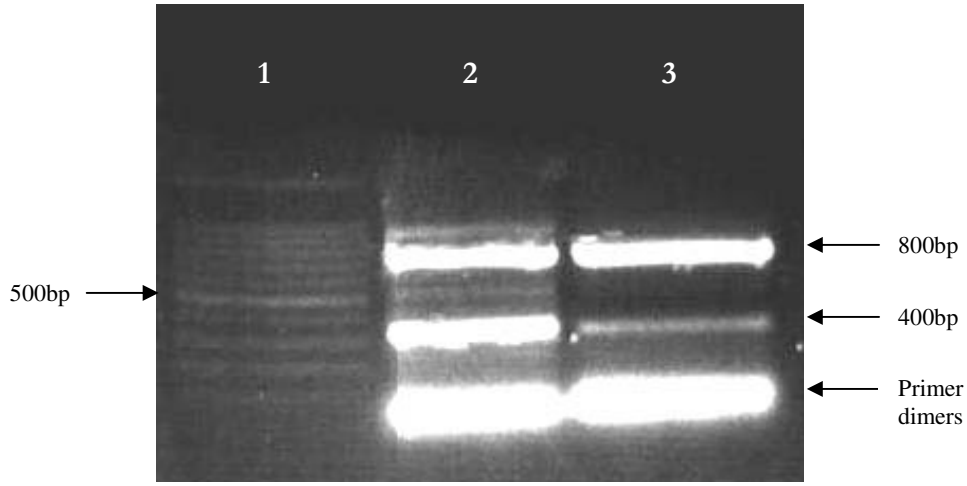

Figure 1 Mitochondrial DNA cytochrome *b* (cyt *b*) gene amplification of dung samples stored at -20°C and extracted following Boom *et al*. (1990). Amplifications were visualised on a 1% agarose gel. Lanes: 1 = 100 bp DNA ladder, 2 = cyt *b* Sample 1, 3 = cyt *b* Sample 2. The 400 bp and 800 bp bands were sequenced. The 500 bp band of the 100 bp DNA ladder is clearly indicated, as are the primer dimers.

```
Query  14   TATCCTATGAAGGCGGTGGCTATGGTGATTAGTAGTAATATAATGCCGGTNTTTCAAGTT  73
            ||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  392  TATCCTATGAAGGCGGTGGCTATGGTGATTAGTAGTAATATAATGCCGGTATTTCAAGTT  333

Query  74   TCCGAGTATAGGTAGGACCCATAGTAGATGTTTCGTCCAATGTGTGTGTATAGGCAGAGA  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  TCCGAGTATAGGTAGGACCCATAGTAGATGTTTCGTCCAATGTGTGTGTATAGGCAGAGA  273

Query  134  AAGAAAATGGATGCTCCGTTTGAGTGTAGTTGTCGAATAATTCAGCCGTAGTTCACATCT  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  AAGAAAATGGATGCTCCGTTTGAGTGTAGTTGTCGAATAATTCAGCCGTAGTTCACATCT  213

Query  194  CGGCAAATATGGGATATAGATGAAAATGCAGTTATTGTGTCAGGTGTATAATGTATGGCT  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  CGGCAAATATGGGATATAGATGAAAATGCAGTTATTGTGTCAGGTGTATAATGTATGGCT  153

Query  254  AGGAATAATCCTGTTAGGATCTGGGTAATTAGGCATGCTCCTAGTAGTGAGCCGAAATTT  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  AGGAATAATCCTGTTAGGATCTGGGTAATTAGGCATGCTCCTAGTAGTGAGCCGAAATTT  93

Query  314  CATCATGCTGAGATGTTGGA  333
            ||||||| ||||||||||||
Sbjct  92   CATCATGTTGAGATGTTGGA  73
```

Figure 2 Alignment result from the BLAST search (Altschul *et al*. 1997) of the 400 bp fragment sequence from the mitochondrial DNA cytochrome *b* (cyt *b*) amplification products. 'Query' refers to the 400 bp sequence and 'Sbjct' refers to the *Loxodonta africana* haplotype DRC11 of the cyt *b* gene obtained on the GenBank database (GenBank accession number: AY741078 - http://www.ncbi.nlm.nih.gov). This GenBank sequence is one of the nine sequences that showed the highest bit score of 620 and an E-value = 6e-175 when aligned to the 400 bp sequence. Numbers to the left and right refer to the position in the DNA sequence. The vertical line between the two sequences indicates the similarities between the sequences and no lines indicate differences.

```
Query  198  CAGNATAAGTGTTCACCACTGNNGAACTCTTTTTATNTTTTCTAGATTTTTCATTGCTGA  257
            ||| |||||| ||||||||||   |||||||||||| ||||||||||||||||||||||
Sbjct  101  CAGTATAAGTCTTCACCACTGTAAAACTCTTTTTATATTTTCTAGATTTTTCATTGCTGA  160

Query  258  GNGGCATAACTATaaaaaaaaaGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCAT  317
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161  GTGGCATAACTATAAAAAAAAAAGTTAAAACTTTCAACAACGGATCTCTTGGCTCTAGCAT  220

Query  318  CGATGAANAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC  377
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  221  CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC  280

Query  378  GGAATCTTTGAACGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGT  437
            | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  281  G-AATCTTTGAACGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGT  339

Query  438  CATTTCACCACTCAAGACCTCTTTTGTTCTTGGCGTTGGAGGTCCTGTTCTCCCCTGAAC  497
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  CATTTCACCACTCAAGACCTCTTTTGTTCTTGGCGTTGGAGGTCCTGTTCTCCCCTGAAC  399

Query  498  AGGCCGCCGAAATGTATCGGCTGTTATACTTGCCCAACTCCCCTGTGTAGTATAAAATTT  557
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  AGGCCGCCGAAATGTATCGGCTGTTATACTTGCCCAACTCCCCTGTGTAGTATAAAATTT  459

Query  558  CTAATTTTTACACTTTGAAGTTCTTGTGTAACACGCCGCTAAACCCTCAACTTTTGTTGA  617
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  460  CTAATTTTTACACTTTGAAGTTCTTGTGTAACACGCCGCTAAACCCTCAACTTTTGTTGA  519

Query  618  ATCTTTCACAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCA  677
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  520  ATCTTTCAC-AAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCA  578

Query  678  ATA  680
            |||
Sbjct  579  ATA  581
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Figure 3 Alignment result from the BLAST search (Altschul *et al*. 1997) of the 800 bp fragment sequence from the mitochondrial DNA cytochrome *b* amplification products. 'Query' refers to the 800 bp sequence and 'Sbjct' refers to the *Ceratocystis fimbriata* strain CMW9572 internal transcribed spacer 1, 5.8S ribosomal RNA and internal transcribed spacer 2 genes obtained on the GenBank database (GenBank accession number: AY233863 - http://www.ncbi.nlm.nih.gov). This GenBank sequence is one of the five sequences that showed the highest bit score of 821 and an E-value = 0 when aligned to the 800 bp sequence. Numbers to the left and right refer to the position in the DNA sequence. A dash (-) within the sequence indicates an insertion or a deletion. The vertical line between the two sequences indicates the similarities between the sequences and no lines indicate differences.

```
Cerato   ??????????????????????????????????????????????????????????????????????????????????????????????????????????????
Ele800   GNCGANGNCNCNNNCNNACNNNNCCCNNNNCCNCGNGNNNNCGNCNTNNNCNNNATCNAGGGGAGGNNCCCNCNCCCNNTTNNTTCATNCCNAGACA

Cerato   TACTCTATA-AACCATGTGTGACATACCCTATCTTGTAGTGAAGATGAATGCTGTTTTGGTGGTTAGGGCCCTTCTGAAAGGGGGGGC--ACCGCTG
Ele800   NNGCNTGTAGAAGCCGGTNTGGACNNCCCTAN-TTGGGNGGNAGATGAATGCNGTTTTGGTTGTTNGGGCCCTNCTGAAANGGGGGGGCGGGGGGTG
              * ** ** *  ** **     *****  ***    * ********** ******** *** ******* ****** ******      * **

Cerato   CCAGCAGTATAAGTCTTCACCACTGTAAAACTCTTTTTATATTT-CTAGATTTTTCATTGCTGAGTGGCATAACTATAAAAAAAAAGTTAAAACTTT
Ele800   NCATCAGNATAAGTGTTCACCACTGNNGAACTCTTTTTATNTTTTCTAGATTTTTCATTGCTGAGNGGCATAACTATAAAAAAAAAGTTAAAACTTT
          ** *** ****** **********   ************ *** ******************** ******************************

Cerato   CAACAACG-ATCTCTTGGCTCTAGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT-CAGAATTCAGTGAATCATCG-AATCTTTG
Ele800   CAACAACGGATCTCTTGGCTCTAGCATCGATGAANAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGGAATCTTTG
         ******** ************************ ********************************** ******************** ********

Cerato   AACGCACATTGCGCCTGGCAGTATTCTGCCAG-CATGCCTGTCCGAGCGTCATTTCACCACTCAAGACCTCTTTTGTTCTTGGCGTTGGAG-TCCTG
Ele800   AACGCACATTGCGCCTGGCAGTATTCTGCCAGGCATGCCTGTCCGAGCGTCATTTCACCACTCAAGACCTCTTTTGTTCTTGGCGTTGGAGGTCCTG
         ******************************** ************************************************************ *****

Cerato   TTCTCCCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTTGCCCAACTCCC-TGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCT
Ele800   TTCTCCCCTGAACAGGCCGCCGAAATGTATCGGCTGTTATACTTGCCCAACTCCCCTGTGTAGTATAAAATTTCTAATTTTTACACTTTGAAGTTCT
         ************************************************** ****************************************

Cerato   TGTGTAACACGCCGCTAA-CCCTCAACTTTTGTTGAATCTTTCACAA-GGTTGACCTCGGATCAGGTAGGAATACCCG-TGAACTTAAGCATATCAA
Ele800   TGTGTAACACGCCGCTAAACCCTCAACTTTTGTTGAATCTTTCACAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA
         ****************** **************************** ****************************** ******************

Cerato   TA??????????????????????????????????????????????????????????????????????????????????????????????????
Ele800   TAAGCNGGAGGAGANNNCNNCGNNCANGNCNNGCGCNCNCGCNCCCGCNANGGCANANNNTAGAGGGGGCCCNCCGNCNCNNCGNNTCTNTCNTCNN
         **

Cerato   ??????????
Ele800   TNNNGGCGCC
```

Figure 4 ClustalX alignment of the 800 bp fragment sequence from the mitochondrial DNA cytochrome *b* amplification products and *Ceratocystis fimbriata* strain CMW9572 internal transcribed spacer 1, 5.8S ribosomal RNA and internal transcribed spacer 2 genes (GenBank accession number: AY233863). 'Cerato' refers to the *C. fimbriata* sequence and 'Ele800' refers to the 800 bp fragment. A question mark (?) indicates missing information, a dash (-) indicates an inferred insertion/deletion event, a star (*) indicates an invariant position and an 'N' indicates an unknown nucleotide.

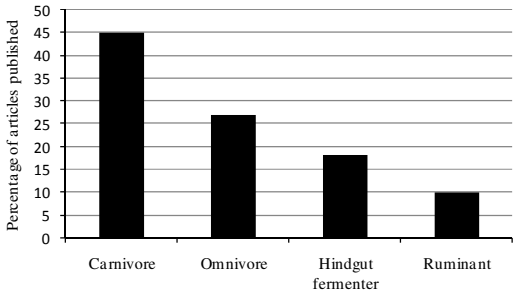

Figure 5 Percentage of articles published, per dietary guild, in *Molecular Ecology* and *Conservation Genetics* using faecal DNA from 1992 to 2009 (see Appendix 7). The dietary guilds include carnivores, omnivores, hindgut-fermenting herbivores and ruminant herbivores.

Discussion

Given the results of this pilot study, elephant faeces were found not to be a reliable source of DNA for further molecular DNA analyses. PCR amplification of both mtDNA and microsatellite loci from elephant dung using elephant-specific primers were not reproducible, despite initial success at DNA extraction.

Storage of elephant dung in DMSO/saturated NaCl solution (Amos & Hoelzel 1991) has been used successfully for other elephant population studies (Nyakaana & Arctander 1999; Eggert *et al*. 2002; Nyakaana *et al*. 2002; Fernando *et al*. 2003; Okello *et al*. 2005; Eggert *et al*. 2008; Gobush *et al*. 2009) and has been recommended as a dung storage method for many other species (baboon (*Papio cynocephalus ursinus*) Frantzen *et al.* 1998; and brown bear (*Ursos arctos*), Murphy *et al.* 2002). DMSO/saturated NaCl solution is a successful storage medium since salt is known to be a good preservative and DMSO increases cellular permeability and thus allows the penetration of salt into cells (Amos & Hoelzel 1991). An added advantage is the wide use of this medium as a storage medium for tissue samples for genetic studies, thus there is no increased expense in storing dung in this medium.

The storage of samples in 70% to 100% ethanol has been used successfully in elephant genetic studies (Fernando & Lande 2000; Fernando *et al*. 2000; Fernando *et al.* 2003, Okello *et al*. 2005) and other species (bonobos (*Pan paniscus*), Gerloff *et al*. 1995; Malayan sun bears (*Helarctos malayanus*) and North American black bears (*Ursus americanus*), Wasser *et al*. 1997; Alpine ibex (*Capra ibex*) and Corsican mouflon (*Ovis musimon*), Maudet *et al*. 2004; Stellar sea lions (*Eumetopias jubatus*) Deagle *et al*. 2005). Despite this, Wasser *et al.* (1997) found that after seven weeks of storage in 70% ethanol

both a 246 bp and 398 bp fragment of mtDNA could not be amplified and Frantzen *et al*. (1998) found that mtDNA was amplified in 92% of samples stored in 70% ethanol, whereas nuclear DNA was amplified from only 27% of these samples. Similarly, Santini *et al*. (2007) found that only 55% of samples stored in 95% ethanol at room temperature successfully amplified six microsatellite loci. Fernando *et al*. (2003) commented that the ratio of preservative to sample is crucial since DNA degradation continues in the presence of insufficient preservative, and this is the most likely reason for the dung samples stored in ethanol in this study being unsuccessful.

Storage of samples at -20°C, in this study, did allow some DNA to be extracted. Frantzen *et al.* (1998) found that freezing of samples at -20°C allowed amplification of mtDNA and nuclear DNA at similar ratios to that of storage in 70% ethanol. Similarly, Wasser *et al.* (1997) found that after four weeks of storage at -20°C samples failed to amplify a 700 bp mtDNA product and failed to amplify several microsatellite loci. Although DNA is likely to be better preserved at lower temperatures, this only retards the rate of DNA degradation (Lindahl 1993; Hofreiter *et al.* 2001), and one is still likely to obtain only degraded fragments of DNA, resulting in no PCR-amplifiable DNA.

Samples that have dried quickly contain almost no moisture, thus DNA degradation by hydrolysis or nucleases is slowed down tremendously (Hofreiter *et al.* 2001; Fernando *et al.* 2003). Several researchers (see Wasser *et al.* 1997) advise the use of silica beads for drying samples, but there are problems associated with this storage method such as fungal contamination as experienced in this study. Murphy *et al.* (2002) found that samples stored with silica beads moulded and took up to 10 days to dry. These samples had the poorest amplification results of all the storage methods they analysed. The ratio of silica beads to sample (Wasser *et al*. 1997), as well as the moisture content of the sample (Murphy *et al.* 2002) is again crucial for good preservation.

Although extractions that showed DNA smears on agarose gels were considered successful, the DNA smears may have contained a large amount of bacterial, fungal and plant DNA which was co-extracted with elephant DNA during the extraction process (Gerloff *et al.* 1995). To date there is no single DNA extraction protocol that is used as a standard protocol for faecal DNA analysis (Eggert *et al*. 2005; Beja-Pereira *et al*. 2009). Most studies tend to use commercially available DNA extraction kits (for example the QIAamp DNA stool kit) or silica particles which bind DNA in the presence of GuSCN (Boom *et al.* 1995). Both of these protocols were successful in this study. Various DNA extraction protocols have been used in other elephant studies, these include: (1) digestion with SDS/Proteinase K followed by extraction with phenol/chloroform/isoamyl alcohol and purification of extracts with Qiagen spin columns (Fernando & Lande 2000; Fernando *et al.* 2000; Fernando *et al.* 2003), (2)

digestion with SDS/Proteinase K followed by extraction via standard phenol/chloroform extraction (Nyakaana & Arctander 1999; Nyakaana *et al.* 2002), (3) GuSCN/silica particle DNA extraction (Eggert *et al.* 2002), or (4) extraction with DNeasy Tissue Kit (Qiagen) (Okello *et al.* 2005). The Flagstad *et al.* (1999) protocol modified to have a final phenol/chloroform extraction step is very similar to the extraction protocol used by Fernando *et al.* (2000; 2003), but excludes the purification of extracts. This protocol was unsuccessful across all storage methods in this study. The purification step in this protocol is crucial, since this allows the removal of contaminants and PCR inhibitors (Ernest *et al.* 2000; Murphy *et al.* 2002; Eggert *et al*. 2005). Although the standard phenol/chloroform extraction used in this study did provide some DNA, this protocol may still need a further purification step to remove some of the PCR inhibitors. The Saiki (1990) protocol was unsuccessful in this study across all storage methods. In all likelihood simply boiling in water for 5 min may not necessarily lyse enough cells for the release of DNA and water may result in continued DNA degradation via hydrolysis (Eggert *et al.* 2005). The Valsechi (1998) protocol fared much better than the Saiki (1990) protocol. This is likely due to boiling for longer in the presence of a lysis buffer. The boiling of samples in water for 20 min (Reed *et al*. 1997 - Method A) showed some success, highlighting the fact that samples need to be boiled for more than 15 min to provide PCR-ready DNA. Further extraction of both the Reed *et al.* (1997 - Method A) and Valsechi (1998) extracts using phenol / chloroform may have removed some contaminants and PCR inhibitors, but a purification step may still be necessary. Protocols isolating DNA with Chelex-100 (Paxinos *et al.* 1997; Reed *et al.* 1997 - Method B) showed some success. The Chelex resin chelates metal ions, thus preventing DNA degradation during boiling (Walsh *et al.* 1991). Further extraction with phenol/chloroform (Reed *et al*. 1997 - Method B) may have removed some contaminants and inhibitors, but again further purification may be necessary. The Launhardt *et al*. (1998) protocol is one of the more successful extraction protocols. The success of this protocol is likely due to the addition of CTAB to the lysis buffer. CTAB is generally used for plant DNA extraction (Doyle & Doyle 1990; Csaikl *et al*. 1998) and allows the removal of PCR inhibitors that may be extracted from plant material present in herbivore faeces (Eggert *et al*. 2005). CTAB has been used for DNA extraction from other herbivore faeces (Huber *et al.* 2002).

The greatest problem facing PCR amplification from dung samples is the problem of PCR inhibitors. Many of these PCR inhibitors originate from polysaccharides, alkaloids, pigments and other substances from the plant material present in the faeces (Flagstad *et al*. 1999; Morin *et al*. 2001; Fernando *et al*. 2003; Okello *et al.* 2005). Another potential factor affecting the quality of DNA obtained from elephant dung is that elephants are hindgut-fermenting herbivores (Feldhamer *et al.* 1999; Willmer *et al.* 2000; Sukumar 2003). In hindgut-fermenters fermentation of plant material occurs once it has passed the stomach and the small intestine, thus micro-organisms involved in

fermentation and the micro-organism's products are not digested and thus are present in the faeces (Feldhamer *et al.* 1999; Willmer *et al.* 2000). This may result in faster DNA degradation by bacteria, compared to degradation of faeces from ruminant herbivores and may result in more PCR inhibitors being present in the faeces. The problems caused by hindgut-fermentation may explain the limited number of published faecal DNA studies of animals such as zebras, rhinoceroses, elephants, lagomorphs, rodents, koalas and wombats compared to studies of ruminant herbivores, omnivores and carnivores. Although Broquet *et al*. (2007) found that diet did not have an effect on amplification success; their review only involved published studies, i.e. studies where DNA extractions from dung had been successful. However, a review of articles published, per dietary guild, indicates that less than a third of these studies have used herbivore faeces (Fig. 5, Appendix 7); highlighting the possibility that DNA studies using faeces from hindgut-fermenters are not as successful as studies on carnivores and are thus not published.

Initial amplifications of mtDNA were successful, but chance amplification of fungal DNA with universal cyt *b* primers highlights the problem of environmental contamination as well as the problem of co-amplification of non-target DNA. Bradley and Vigilant (2002) amplified DNA sequences that resembled the DNA sequences of several bacteria when amplifying microsatellite loci from gorilla faeces. Similarly, in this study, sequences were obtained which had the highest percentage identity to fungal DNA, *Ceratocystis fimbriata*. *Ceratocystis fimbriata* is known to be a plant pathogen of a wide variety of hosts including sweet potato (Witthuhn *et al.* 1999), which is likely to be included in the diet of Zoo elephants (A. Tordiffe, National Zoological Gardens, Pretoria, personal communication) and may have been infected before or after digestion. But, the amplified DNA may by chance alone have been similar to that of *C. fimbriata* internal transcribed spacer 1 genes. This fact is highlighted by the ClustalX alignment of the two sequences.

Failure to amplify cyt *b* three months after extraction may be due to DNA degradation of extracts, since all extracts were eluted in water, which allows degradation by hydrolysis. Several freeze-thaw cycles of extracts would have further degraded the DNA (Eggert *et al.* 2005). Elephant specific D-loop primers were used in an attempt to lower the possibility of amplifying non-target DNA. The optimisation of D-loop PCR conditions is difficult with low quantities of DNA and the conditions attempted before contamination did not approach the optimal temperature found for amplification of tissue samples (see Chapter 4). Although some extraction techniques had no amplification product, primer dimers were seen. The presence of primer dimers indicates that *Taq* polymerase was not inhibited (Reed *et al.* 1997). PCR inhibition may have been decreased by the addition of betaine to the PCR cocktail. Betaine has been found to increase DNA amplification, improves the consistency of

amplifications (Baskaran *et al.* 1996; Pergams *et al.* 2003) and is often used in amplification of ancient DNA (Mulligan 2005). Amplifications of microsatellite loci are likely to experience the same problems as that of mtDNA amplifications. Further problems include very low quantities of DNA, in comparison to mtDNA, since nuclear DNA is only present as a single copy within a cell (Taberlet *et al.* 1999). Over time the degradation of nuclear DNA may continue to a point where PCR amplification will be impossible (Wasser *et al.* 1997).

Factors that could have improved success

Several products have been used in other studies to improve PCR success, but were not used in this study. Extracts could have been purified with the use of commercially available purification columns. BSA could have been added to the PCR cocktail. BSA overcomes inhibition by substances that may have been co-extracted with the DNA (Pääbo 1990; Kohn & Wayne 1997; Fernando *et al*. 2003; Okello *et al.* 2005). A higher fidelity *Taq* polymerase (for example Expand High Fidelity Taq, Applied Biosystems) or *Taq* polymerase that only becomes active after a 10 minute incubation at 95°C (for example AmpliTaq Gold™, Applied Biosystems) could have been used to improve PCR fidelity as well as reduced non-target amplifications (Taberlet *et al.* 1999; Beja-Pereira *et al*. 2009). Hot start PCR could have been used to maximise PCR robustness (Eggert *et al.* 2005). Aerosol-resistant filter tips could have been used during PCR set up. Lastly, the use of a separate room for extractions and PCR preparation would have improved success (Taberlet *et al*. 1999; Beja-Pereira *et al*. 2009) but due to space and financial constraints it was impossible. Further techniques that could have improved success include repeated DNA extractions per method per sample, and multiple PCR amplifications per sample (Taberlet *et al.* 1996; Ernest *et al.* 2000; Fernando *et al.* 2003). Time and financial constraints prevented the use of these techniques for this study.

Is faecal DNA viable for a population genetic study?

An advantage of obtaining DNA from faeces is that animals do not need to be disturbed or sacrificed to obtain samples (Kohn & Wayne 1997; Taberlet *et al.* 1999; Fernando *et al.* 2003, Okello *et al.* 2005). Information regarding population structure, reproduction and sex ratios can be obtained from faecal DNA (Kohn & Wayne 1997; Reed *et al*. 1997; Taberlet *et al*. 1997; Kohn *et al*. 1999; Ernest *et al*. 2000; Farrel *et al*. 2000; Vidya *et al*. 2005a; Piggott *et al.* 2006; Eggert *et al*. 2008; Gobush *et al*. 2009). However, there are many problems associated with this method, as highlighted in this study. Several reagents that are not used in conventional molecular population studies need to be bought (Pääbo 1990; Kohn & Wayne 1997; Taberlet *et al.* 1999; Fernando *et al*. 2003; Okello *et al.* 2005), multiple DNA extractions from a single sample need to be carried out, and multiple PCR amplifications of the same primer for each sample need to be performed (Taberlet *et al.* 1996; Ernest

et al. 2000; Fernando *et al.* 2003). These factors may result in an increase in expense as well as analysis time. Another even more crucial point is the possibility of obtaining genotyping errors. A high percentage of genotyping errors will result in incorrect conclusions from the data (Taberlet *et al*. 1996; Kohn & Wayne 1997; Taberlet *et al*. 1997; Goosens *et al*. 1998; Taberlet *et al.* 1999; Bradley & Vigilant 2002; Murphy *et al*. 2002; Creel *et al*. 2003; Fernando *et al*. 2003; Murphy *et al*. 2003; Okello *et al*. 2005; Beja-Pereira *et al*. 2009). I believe that the use of faecal DNA to study small elephant populations is currently not financially viable. Although the majority of elephants can be individually identified, such that obtaining dung from known individuals is possible, biopsy darting of individuals is just as possible. The possibility that biopsy darting will disturb the 'natural' behaviour of the elephants is a valid concern (G. van Dyk, North-West Parks and Tourism Board, Mafikeng, personal communication; T. Burke, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, personal communication), but I believe that it is likely to only cause a disturbance for a few hours (A.M. Whitehouse, Terrestrial Ecology Research Unit, Department of Zoology, University of Port Elizabeth, personal communication; Delsink *et al*. 2007; Burke *et al*. 2008) and is not likely to result in any long-term behavioural changes.

In conclusion, this study showed that obtaining DNA of a good enough quality and quantity from elephant dung was not possible. Better results may be obtained with improved methods. The advantage of obtaining faecal DNA should be weighed against the various disadvantages of faecal DNA as discussed above.

Chapter 4[3]

Conservation genetics of small populations of elephants in South Africa

Abstract

Most elephant populations in South Africa, excluding the Kruger National Park (KNP) and the Addo Elephant National Park (AENP), consist of originally small numbers of reintroduced individuals. Small populations are more likely to suffer from the loss of genetic diversity and inbreeding, which may result in an increase in extinction risk. Historically these factors have not been considered as part of elephant translocations and management. The genetic diversity of managed populations should be assessed and evaluated to determine successful population establishment and future population persistence. Microsatellite diversity in the populations of Pilanesberg National Park, Madikwe Game Reserve, Phinda Private Game Reserve and Hluhluwe-iMfolozi Park were determined, and compared to the KNP and the AENP populations. Additionally, both microsatellite and mitochondrial DNA (mtDNA) control region diversity within the southern African region were investigated and compared to the diversity of eastern African populations. Microsatellite genetic diversity of the reintroduced populations showed similar levels of heterozygosity and greater allelic richness than that of the KNP population. As in previous studies, the AENP population not only had a lower genetic diversity, but was significantly differentiated from other southern African populations due to a severe bottleneck suffered by the AENP population. MtDNA diversity was within the range observed in other African elephant populations. MtDNA haplotypes were shared between southern and eastern Africa, but, the two distinct haplogroups found in previous studies could not be identified in our data set. Although our results indicate genetically healthy populations, future management plans should take genetic diversity into consideration so as to minimise the potential genetic threats that these small populations could experience.

[3] This chapter will be shortened for submission to African Zoology. Authorship will be as follows: Alves Ferreira MA, Slotow R and Bloomer P.

Introduction

Many of the conservation areas outside of national parks in South Africa consist of small areas of land, generally less than 1000 km^2, which were reclaimed from areas that were previously used for agriculture (Hall-Martin 1992). Since 1979 well over 800 elephants (*Loxodonta africana*) have been introduced into 58 conservation areas (Garaï *et al*. 2004). In 2003 the largest of these reintroduced populations numbered 387 individuals in a fenced area of approximately 900 km^2 (Hluhluwe-iMfolozi Park (HiP), Mackey *et al.* 2006) and one of the smallest populations numbered 10 individuals in a fenced area of approximately 80 km^2 (Mabula Game Reserve, Mackey *et al*. 2006). These population sizes are in stark contrast to more 'natural' elephant populations, which number in the thousands (Slotow *et al*. 2005; Mackey *et al*. 2006).

The longevity of elephants makes the short term analysis of conservation genetic considerations difficult. Recent studies of small elephant populations in South Africa show that females reach sexual maturity between the ages of eight and 12 years and can have an intercalving period of between two and seven years (Mackey *et al.* 2006; Chapter 2) with a gestation period of 22 months. Previous and current management (for example: selection of founding individuals, and contraception (Delsink *et al*. 2006), and the removal of problem animals (Slotow *et al*. 2000) may have had, or is having, an ongoing effect on genetic diversity within populations. This is of particular concern when one considers the translocation history of elephants in South Africa. Many of the populations are sourced from a single population, the Kruger National Park (KNP) population, with small founder numbers (mean founder size = 26.4 for 57 reserves studied, Garaï *et al*. 2004), and female-skewed sex ratios (Whyte 2001; Garaï *et al*. 2004; Slotow *et al.* 2005; Mackey *et al*. 2006). All of these populations are fenced-in and essentially act as islands with no immigration or emigration (Slotow *et al*. 2005).

These reintroduced elephant populations are small populations which can be affected by founder effect, population bottlenecks, and a lack of exchange of genetic material between populations which can result in genetic drift and affect genetic diversity (Frankham *et al.* 2002; DeSalle & Amato 2004; Frankham 2010b). For example, tusklessness in the Addo Elephant National Park (AENP) has been proposed to be due to a founder effect and genetic drift (Whitehouse 2001). Inbreeding depression can result in a reduction in reproduction and survival (Ralls *et al*. 1988) and has been documented in an introduced population of lion in HiP (Trinkel *et al*. 2008), and other free-ranging species (see examples in Hedrick & Kalinowski 2000; Keller & Waller 2002; Frankham 2003; Frankham 2010a). The loss of genetic diversity not only affects the current population and its response to stochastic

events (for example: Coltman *et al*. 1999; Rijks *et al*. 2008), but also affects its ability to adapt to future changes (Lacy 1997; Frankham 2005).

The evolutionary processes which have resulted in the biological diversity we currently see can be identified by genetic and ecological analyses ((Moritz 1999; Crandall *et al.* 2000; Moritz 2002). Moritz (1999) suggested the use of the evolutionary significant unit (ESU) and management unit (MU) concepts for delineating populations which may be regionally differentiated, and thus may require protection (Crandall *et al*. 2000, Moritz 2002). ESUs are groups of populations or MUs that are reproductively isolated and have developed adaptive differences allowing these groups to be managed separately (Moritz 1999; Crandall *et al*. 2000). MUs are demographically independent populations where migration between populations is sufficiently low to allow for the monitoring and management of each unit independently, although limited mixing of MUs is allowed (Moritz 1999; Palsbøll *et al*. 2007).

In order to evaluate whether genetic issues affecting small populations, such as founder effect, population bottlenecks, genetic drift and inbreeding/inbreeding depression, are relevant to reintroduced elephants, the genetic diversity of these small populations needs to be assessed (Frankham *et al*. 2002). Genetic diversity can be determined using a variety of molecular genetic markers. Microsatellite loci have high mutation rates which result in high levels of polymorphism (Amos *et al*. 1993; Schlötterer 2000), they are codominant (Litt & Luty 1989; Jarne & Lagoda 1996), are inherited in a mostly Mendelian fashion (Litt & Luty 1989; Jarne & Lagoda 1996; Dakin & Avise 2004; Selkoe & Toonen 2006), and are selectively neutral (Tóth *et al*. 2000; Li *et al*. 2002), making them ideal markers for population genetic studies (Bruford & Wayne 1993; Beaumont & Bruford 1999; Zhang & Hewitt 2003; Selkoe & Toonen 2006; Sarre & Georges 2009). The mitochondrial DNA (mtDNA) control region is another marker that is widely used in genetic studies (Avise *et al*. 1987; Harrison 1989; Avise 1994; Taberlet 1996) due to the fact that this extra-nuclear, closed-circular molecule is maternally inherited and usually does not undergo recombination (Brown 1985; Moritz *et al*. 1987; Harrison 1989; Birky 2001; Rokas *et al*. 2003). In addition the evolutionary rate over the whole mtDNA molecule is five to 10 times greater than that of single copy nuclear DNA (scnDNA, Brown *et al*. 1979; Ferris *et al*. 1983; DeSalle *et al*. 1986; Moriyama & Powell 1997). The mtDNA control region is a non-coding segment of mtDNA (Brown *et al*. 1993; Hoelzel 1993; Taberlet 1996), with properties of maternal inheritance, non-recombination and a mutation rate four to five times faster than the entire mtDNA molecule, making it a useful marker for population genetic and evolutionary studies (Harrison 1989; Brown *et al*. 1993; Ballard & Whitlock 2004; Avise 2009).

Genetic studies on other African elephant populations have used microsatellites and the mtDNA control region sequence variation to look at population structure and genetic diversity (Nyakaana & Arctander 1999; Nyakaana *et al*. 2001; Nyakaana *et al.* 2002; Archie *et al*. 2006; Archie *et al*. 2007; Eggert *et al*. 2008; Ishengoma *et al*. 2007; Archie *et al*. 2008; Okello *et al*. 2008a, b), parentage (Whitehouse & Harley 2001; Ishengoma *et al*. 2007; Archie *et al*. 2007; Hollister-Smith *et al*. 2007), bottlenecks (Whitehouse & Harley 2001), forensics (Wasser *et al*. 2004; Wasser *et al*. 2007), phylogeography (Comstock *et al*. 2002; Eggert *et al*. 2002), and the savanna/forest elephant species debate (Roca *et al*. 2001; Debruyne 2005; Roca & O'Brien 2005; Johnson *et al.* 2007; Roca *et al*. 2007). Many of these studies have tried to provide guidelines for the management of the species as a whole, however, the difficulty in delineating whether there are two or three elephant 'taxa' (Eggert *et al*. 2002; Nyakaana *et al*. 2002), has not allowed geneticists to provide concise management guidelines. More in-depth studies, at the regional and population level, have warned against the potential effects of a loss in genetic diversity due to genetic drift, founder effect, inbreeding (and its concomitant inbreeding depression), or the disruption of social groups (as a result of poaching); advocating the use of genetic data when developing population management plans, as well as, providing support for conservation efforts that stop poaching (Nyakaana *et al*. 2001; Whitehouse & Harley 2001; Archie *et al*. 2008; Okello *et al*. 2008).

The KNP, the AENP and the Knysna elephant populations are the only South African elephant populations for which genetic studies using microsatellites and/or the mtDNA control region have been published (Whitehouse & Harley 2001; Comstock *et al*. 2002; Eggert *et al*. 2002; Nyakaana *et al*. 2002; Wasser *et al*. 2004; Eggert *et al*. 2008; Wasser *et al*. 2007). A microsatellite study on the genetic diversity of the AENP population found that there was a significant reduction in the genetic diversity in this population, both in allele numbers and heterozygosity, in comparison to the KNP population (Whitehouse & Harley 2001). Whitehouse and Harley (2001) observed high levels of genetic differentiation between the AENP and KNP populations, suggesting rapid genetic drift of the AENP population due to its small population size of 11 individuals after the proclamation of the park in 1931 (Trollope 1931). This study illustrates the stochastic risks associated with translocations, even when founders are sourced from a heterogeneous gene pool. A study on the Knysna elephants detected five individuals in a population that was previously believed to be extinct. Genetic diversity of these individuals was higher than that of AENP, but similar to that of KNP. Furthermore, a single mtDNA control region haplotype was shared among all five individuals and this haplotype matches one previously found in the AENP population (Eggert *et al*. 2008).

The KNP population has lower genetic diversity than three Ugandan populations for the same microsatellite loci (Nyakaana & Arctander 1999; Whitehouse & Harley 2001), and the expected heterozygosities of southern African populations were lower than that of eastern African populations studied (Nyakaana *et al*. 2002). Despite the eastern African elephant populations having suffered severe declines in numbers during the 1970's and 1980's due to ivory poaching (Ottichilo *et al*. 1987) which affected their genetic diversity, southern African elephant populations suffered severely during the eighteenth and nineteenth centuries due to hunting and poaching (Hall-Martin 1992; Skinner & Chimimba 2005) which resulted in a "lasting loss of genetic diversity" in the region (Whitehouse & Harley 2001). Therefore, future management of southern African populations needs to take into consideration the potential problems due to a loss of genetic diversity as a result of founder effects, population bottlenecks, inbreeding, and genetic drift, essentially managing them as small populations and preventing stochastic events from affecting these populations.

A study on restriction site variation of mtDNA in AENP and KNP elephants found that the AENP samples were monomorphic and that the KNP samples showed a moderate degree of polymorphism (0.27%, Essop *et al*. 1996). There are currently no published studies where mtDNA control region diversity has been analysed within the southern African region. Most published studies have looked at mtDNA control region at the continental and inter-regional level (Nyakaana *et al*. 2002; Eggert *et al*. 2002; Johnson *et al*. 2007). On a continental level the elephant mtDNA control region had an average nucleotide diversity of 3.0% (lower than other large African mammals, Nyakaana & Arctander 1999) and haplotype diversity of 0.99 (Johnson *et al*. 2007). Allele network analyses of the mtDNA control region at the continental level have identified three (Nyakaana *et al*. 2002) to four (Eggert *et al*. 2002; Johnson *et al.* 2007) haplogroups among African elephants. Each 'savanna' haplogroup identified in these three studies found highly divergent mtDNA haplotypes to coexist in eastern and southern African populations. Two hypotheses have been put forward to explain this phenomenon: (1) "large long-term effective population sizes" which resulted in incomplete lineage sorting, and (2) "recent secondary population admixture due to range expansion from refugia" (Georgiadis *et al*. 1994; Eggert *et al*. 2002; Nyakaana *et al*. 2002). However, despite Johnson *et al*. (2007) having added haplotypes from central African forest areas, the limited number of samples from the entire range of the African elephant may result in incorrect assumptions of population structure (Debruyne 2005).

The genetic diversity of small populations of introduced elephants needs to be assessed in order to aid future management decisions in their aim to reduce potential genetic threats. The aim of this chapter was to determine the level of microsatellite diversity in the translocated elephant populations of Pilanesberg National Park (PNP), Madikwe Game Reserve (Madikwe), Phinda Private Game Reserve

(Phinda) and HiP, and to compare these values to that of populations in the KNP, the source of many of the founder individuals, and the AENP, a population known to have suffered a severe bottleneck in 1931 (Whitehouse & Harley 2001). Microsatellite and mtDNA control region diversity within the southern African region was investigated to determine whether genetic diversity had been lost by heavily managed populations in South Africa. The effects of historical and recent population reductions on continental-level genetic diversity, and the presence of two distinct savanna mtDNA control region haplogroups, as identified by previous studies (Eggert *et al*. 2002; Nyakaana *et al*. 2002; Johnson *et al*. 2007), were explored.

Materials and methods

Sample Collection

Samples were obtained from various elephant populations in southern Africa (Table 1, Fig. 1). Tissue samples were collected from four game reserves within South Africa: PNP (21 individuals), Madikwe (29 individuals), KNP (10 individuals), and Phinda (16 individuals). Four samples were obtained from Northwestern Maun, Botswana. Tissue samples were obtained from ear biopsies of sedated animals. Samples were stored in tissue/blood storage buffer (0.1 M Tris, 0.1 M EDTA-Na_2, 1.0 M NaCl and 0.5% SDS; modified from Longmire *et al*. 1988) at room temperature. Blood samples were obtained from elephants in HiP (20 individuals) and stored in tissue/blood storage buffer at 4°C. Plucked tail hairs were obtained from elephants in Etosha National Park, Namibia (six individuals); Kafue National Park, Zambia (nine individuals); northwestern Maun, Botswana (one individual); Santawani, Botswana (one individual); Gonorezhou National park, Mana Pools, Mashumbo Pools and Bubi River in Zimbabwe (total of 11 individuals) and stored in separate envelopes at room temperature. DNA extractions from a previous study in the AENP (21 individuals, Whitehouse & Harley 2001) and tissue samples from a current study in the KNP (17 individuals, P. Grobler, Department of Genetics, University of the Free State, personal communication), were provided by the respective researchers. In addition, mtDNA sequences were obtained from a previous study on southern African elephants, including five sequences from Maputo Elephant Reserve (Mozambique), five sequences from Tembe Elephant Park (South Africa) and 10 sequences from KNP (R.J. van Aarde, Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria & T.J. Robinson, Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication). A summary of the history of reintroduced populations in South Africa can be found in Table 2 (see Chapter 1 for further details).

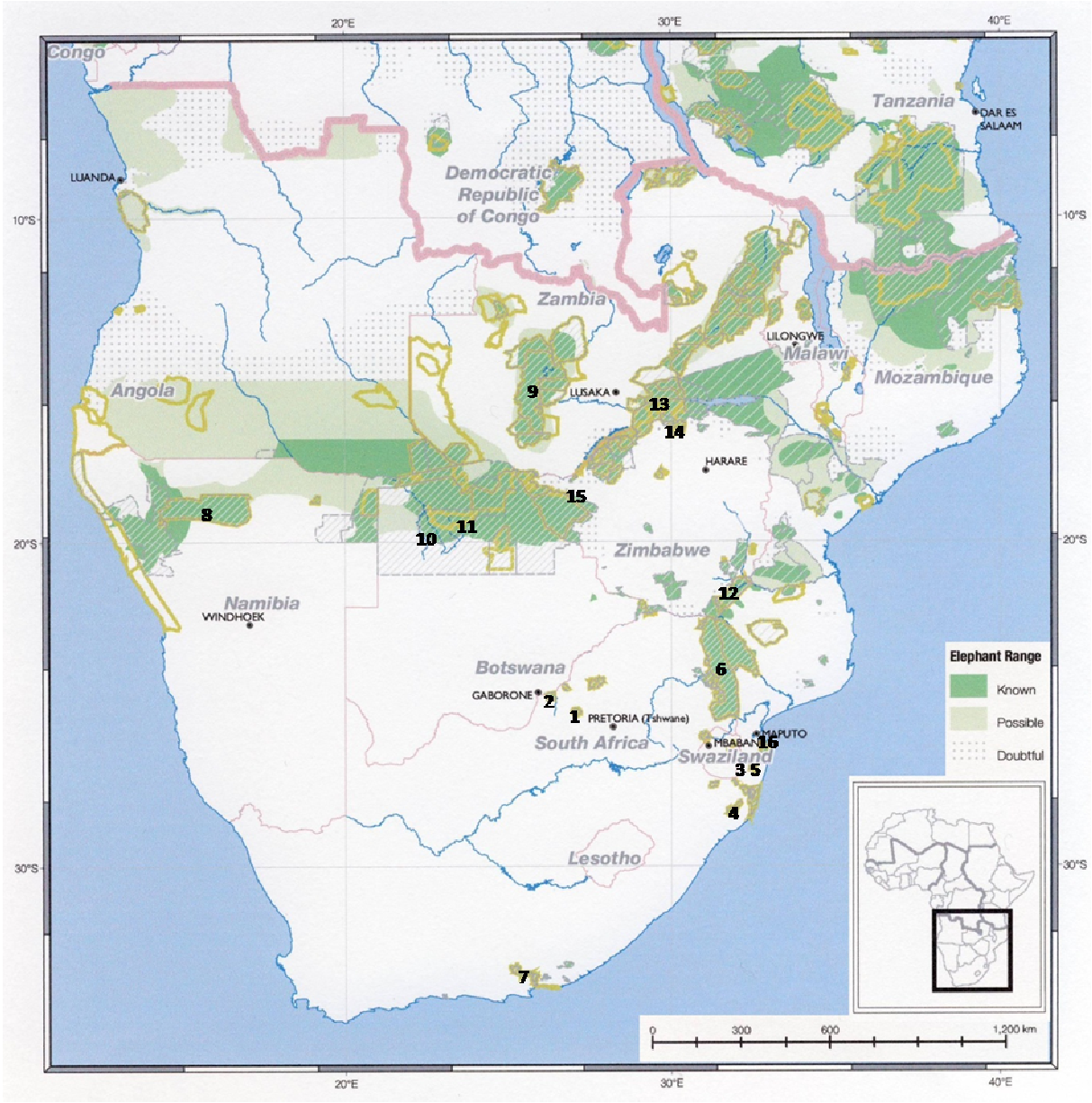

Figure 1 Elephant distribution in southern Africa with sampling localities for this study. 1 – Pilanesberg National Park, 2 – Madikwe Game Reserve, 3 – Phinda Private Game Reserve, 4 – Hluhluwe-iMfolozi Park, 5 – Tembe Elephant Park, 6 – Kruger National Park, 7 – Addo Elephant National Park, 8 – Etosha National Park, 9 – Kafue National Park, 10 – Northwestern Maun, 11 – Santawani, 12 – Gonorezhou National Park, 13 – Mana Pools, 14 – Mashumbo pools, 15 – Bubi River, 16 – Maputo Elephant Reserve; see Table 1 for further details. Map modified from the *African Elephant Status Report 2007* (Blanc *et al*. 2007).

Table 1 Locality, country of origin and sample size of southern African specimens collected or used in this study.

Locality	GPS coordinates	Country	Sample size	Sample type
Pilanesberg NP	25°12'S 27°06'E	South Africa	21	Tissue biopsies
Madikwe GR	24°43'S 26°10'E	South Africa	29	Tissue biopsies
Phinda GR	27°44'S 32°25'E	South Africa	16	Tissue biopsies
Hluhluwe-iMfolozi Park	28°11'S 32°01'E	South Africa	20	Blood samples
Tembe Elephant Park*	27°01'S 32°24'E	South Africa	5	mtDNA sequence
Kruger NP	25°29'S 30°49'E	South Africa	27	Tissue biopsies
Kruger NP*	25°29'S 30°49'E	South Africa	10	mtDNA sequence
Addo Elephant NP	33°30'S 25°44'E	South Africa	21	DNA extractions
Etosha NP	19°07'S 16°18'E	Namibia	6	Tail hairs
Kafue NP	14°25'S 26°23'E	Zambia	9	Tail hairs
Northwestern Maun	19°14'S 22°29'E	Botswana	4	Tissue biopsies
Northwestern Maun	19°14'S 22°29'E	Botswana	1	Tail hairs
Santawani	19°30'S 23°37'E	Botswana	1	Tail hairs
Gonorezhou NP	21°40'S 31°40'E	Zimbabwe	8	Tail hairs
Mana Pools	15°46'S 29°23'E	Zimbabwe	1	Tail hairs
Mashumbo Pools	16°10'S 30°33'E	Zimbabwe	1	Tail hairs
Bubi River	18°55'S 27°43'E	Zimbabwe	1	Tail hairs
Maputo Elephant Reserve*	26°25'S 32°45'E	Mozambique	5	mtDNA sequence

NP = National Park, GR = Game Reserve, mtDNA = mitochondrial DNA.

* Mitochondrial DNA control region sequences obtained from R.J. van Aarde and T.J. Robinson for mitochondrial DNA analysis only.

Table 2 Elephant populations sampled in South Africa, with details of introductions. No data are given regarding introductions for Kruger NP, Addo Elephant NP and Tembe Elephant Park since these populations become established from elephants already present in these parks at the time of proclamation. Gonorezhou NP is found in Zimbabwe.

Reserve	Reserve size	Total introduced[1]	Years introduced	Source population	Current population size (Year)
Kruger NP	19000 km^2			Self	12 427 (2006)†
Addo Elephant NP	1250 km^2			Self	459 (2005)†
Tembe Elephant Park[4]	300 km^2			Self	167 (2005)†
Pilanesberg NP	570 km^2	125	1979 – 1998	Addo Elephant NP[3], Kruger NP	136 (2005)$^{\#}$
Madikwe GR	620 km^2	237	1993 – 1999	Gonorezhou NP, Kruger NP	455 (2005)†
Phinda Private GR	150 km^2	106	1992 – 2003	Gonorezhou NP, Kruger NP	81 (2005)$^{\Delta}$
Hluhluwe-iMfolozi Park	890 km^2	241	1981 – 2000	Kruger NP	387 (2003)*

NP = National Park, GR = Game Reserve.

[1]Total numbers of elephants introduced, including animals which were culled, removed or died. Modified from Mackey *et al.* (2006)

[3]Only one sub-adult male was left in Pilanesberg after the initial translocation from Addo (Anderson 1994).

[4]Only mitochondrial DNA sequences were obtained from this population, see 'Materials and methods'.

Sources: † - Blanc *et al.* 2007, $^{\#}$ - Woolley *et al.* 2008b, $^{\Delta}$ - Druce *et al.* 2008, * - Mackey *et al.* 2006.

DNA Extraction

Total genomic DNA from tissue and hair samples was extracted using the same technique. This involved drying the tissue and finely chopping 20 mg of tissue or the bulb end of a single tail hair (which had been pre-soaked in distilled water). The tissue was digested overnight in 500 µl extraction buffer (0.05 M Tris-HCl, 0.5 M EDTA-Na_2, 1.0 M NaCl, and 10% SDS) and 0.5 mg Proteinase K (Roche Diagnostics). Samples were further digested for 30 min at 37°C with 0.1 mg RNase A (Roche Diagnostics). Digestions were followed by extraction with phenol (repeated twice) after which the phenol was removed using a 24:1 solution of chloroform:isoamyl alcohol (Sambrook *et al.* 1989). DNA was precipitated using 2.5 volumes 96% ethanol and 0.1 volumes 3.0 M sodium acetate (Sambrook *et al.* 1989). The precipitated DNA was pelleted in a micro-centrifuge at 13 000 rpm for 30 min and then washed with 70% ethanol. DNA pellets were air-dried and resuspended in 50-100 µl SABAX® water (Adcock Ingram). DNA from blood samples was extracted using the QIAamp Blood

Kit (Qiagen), according to the manufacturer's protocol. DNA was eluted in the final elution step with 50 µl SABAX® water. The quality of DNA extracts was determined by electrophoresis through 1% agarose gels (Laboratory Specialist Services) and the concentration using fluorometry.

Microsatellite DNA amplification and genotyping

Previously published African elephant microsatellite loci (Nyakaana & Arctander 1998; Comstock *et al.* 2000, Eggert *et al.* 2000) which had been found to be polymorphic in both eastern and southern African elephant populations (Nyakaana & Arctander 1998; Nyakaana & Arctander 1999; Comstock *et al.* 2000; Eggert *et al.* 2000; Whitehouse & Harley 2001; Comstock *et al*. 2002; Eggert *et al*. 2002; Nyakaana *et al*. 2002) were selected for genotyping all the samples. These loci included FH19, FH39, FH67 (Comstock *et al.* 2000), LA5, LA6 (Eggert *et al.* 2000), LafMS02, LafMS03, LafMS04, and LafMS05 (Nyakaana & Arctander 1998). Loci were amplified in 10 µl reaction volumes containing approximately 40 ng of DNA, 2.5 mM $MgCl_2$, 1 x buffer, 2.5 pmol of each primer (fluorescently labelled forward primer - see Table 3), 0.2 units of Super-Therm *Taq* DNA polymerase (Southern Cross Biotechnology), and 0.2 mM dNTPs (Promega). The final volume was made up using SABAX® water. Amplification conditions were 94°C for 3 min; 10 cycles of 94°C for 30 s, annealing temperature (60° - 64°C) for 30 s, 72°C for 30 s, followed by 25 cycles of 89°C for 30 s, annealing temperature (60° - 64°C) for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. See Table 3 for optimal annealing temperatures for each locus. All amplifications were carried out using a GeneAmp 9700 thermal cycler (Applied Biosystems).

Table 3 Microsatellite loci used in determining genetic variation among elephant populations in this study. Optimal PCR annealing temperatures, fluorescent labels (filter set C, Applied Biosystems) used for each microsatellite locus and the volume of PCR product used for electrophoresis on the ABI automated sequencer.

Locus (repeat unit)		**Annealing temperature**	**Fluorescent label**	**Volume used for electrophoresis**
FH19	$(CA)_{15}$	62°C	FAM	2 µl
FH39	$(CA)_{18}$	62°C	FAM	2 µl
FH67	$(CA)_{15}$	60°C	HEX	5 µl
LafMS02	$(AC)_{16}$	60°C	TET	3 µl
LafMS03	$(TG)_{15}$	60°C	TET	3 µl
LafMS04	$(TG)_{14}$	62°C	FAM	1 µl
LafMS05	$(AC)_{11}$	64°C	HEX	1 µl
LA5	$(CA)_{13}$	60°C	HEX	2 µl
LA6	$(CA)_{13}$	64°C	HEX	1 µl

Electrophoresis of each polymerase chain reaction (PCR) product was performed using 2% agarose gels (Laboratory Specialist Services) to determine whether amplification was successful. Negative controls were included during PCR and were visualised on a gel to ensure that no cross-contamination of samples or stock solutions occurred during the setting up of PCR reactions. PCR products which amplified successfully were further tested for polymorphism. As a result of different allele size ranges and the labelling of loci with various fluorescent labels, various loci were co-loaded per gel lane. PCR products were pooled for each animal using the volumes as in Table 3. SABAX® water was added to prevent overloading on the gel. Of this mixture, 1 µl was added to 2.5 µl of a formamide mix (1.5 µl deionised formamide, 0.5 µl GeneScan-500™ TAMRA standard, and 0.5 µl GeneScan loading buffer) (Applied Biosystems). GeneScan-500™ TAMRA acts as an internal lane standard to facilitate determination of allele sizes. The mixture was denatured at 90°C for 2 min before loading and then electrophoresed on a 6% polyacrylamide-sequencing gel on an ABI Prism 377 automated sequencer (Applied Biosystems). Sizes of the electrophoresed PCR products were compared to the TAMRA size standard using the program GeneScan® version 2.0 (ABI Prism, Perkin Elmer Biosystems). Alleles were then scored in the program Genotyper® version 2.0 (ABI Prism, Perkin Elmer Biosystems) using the least-squares minimisation procedure to define allelic sizes ('bins', Idury & Cardon 1997). The 'binning' of alleles involves the grouping of allele fragments of a particular size into a range centred on the average allele size. The information generated by Genotyper was exported to a spreadsheet programme, where the data were converted to an acceptable format for use in statistical and population analyses programmes.

Microsatellite data analysis

In order to determine whether sampling localities could be combined for further analysis, homogeneity tests were performed using CHIFISH (Ryman 2006). This programme tests for allele frequency homogeneity by both the chi-squared method and by using Fisher's method of combining *p*-values obtained by Fisher's exact test (Ryman 2006). *P*-values were determined using 10000 dememorisations, 100 batches and 5000 iterations per batch. Statistical power for these tests was determined using POWSIM (Ryman & Palm 2006).

Linkage disequilibrium across pairs of loci per population and conformance to Hardy-Weinberg equilibrium, globally and across populations, was determined using GENEPOP version 3.3 (Raymond & Rousset 2000). The complete enumeration method (Louis & Dempster 1987) was employed for loci with less than five alleles and the Markov chain method (Guo & Thompson 1992) for loci with five or more alleles. For all comparisons in GENEPOP, the Markov chain was set to 200 batches, 1000 iterations and 1000 dememorisations. Critical significance levels were corrected using Bonferroni tests

(Rice 1989). The presence of null alleles was determined using MICROCHECKER version 2.2.3 (van Oosterhout *et al*. 2004). The Monte Carlo simulation was set to 1000 iterations with a 95% confidence interval.

Various measures of genetic diversity were determined for the groups identified by the homogeneity tests in CHIFISH. The mean expected heterozygosity, mean observed heterozygosity, mean number of alleles per locus, and mean number of alleles per polymorphic locus were calculated using GDA version 1.1 (Lewis & Zaykin 2001). Allelic frequency per locus, private alleles, and monomorphic loci were calculated using Microsatellite toolkit (Park 2001).

Population differentiation can be determined using either F_{ST} or R_{ST} values. F_{ST} assumes the infinite alleles model (IAM, Kimura & Crow 1964; Weir & Cockerham 1984) and R_{ST} assumes the stepwise mutation model (SMM, Kimura & Ohta 1978; Slatkin 1995). Under the IAM a mutation results in an allele previously not found in the population (Kimura & Crow 1964; Estoup *et al*. 2002). Under the SMM there is an equal probability of gaining or losing a tandem repeat, resulting in mutations towards alleles that may already be present in the population (Kimura & Ohta 1978; Estoup et al. 2002). Although most microsatellite loci are believed to follow a SMM (Di Rienzo *et al*. 1994; Ellegren 2000; Schlötterer 2000; Xu *et al*. 2000; Estoup *et al.* 2002; Ellegren 2004), there is much debate regarding which of these two measures of population differentiation should be used (Gaggiotti *et al*. 1999; Lugon-Moulin *et al.* 1999; Balloux & Goudet 2002; Balloux & Lugon-Moulin 2002; Hardy *et al.* 2003). The suitability of F_{ST} versus R_{ST} was determined using allele size permutation tests (Hardy *et al*. 2003) implemented in SPAGeDi version 1.2g (Hardy & Vekemans 2002). This test randomises allele sizes within a locus while maintaining genotypic states of individuals. If allele sizes contribute to genetic differentiation (i.e. following SMM), the R_{ST} computed from the permuted data set (pR_{ST}) would be less than the observed R_{ST} (Hardy *et al*. 2003). Significance was calculated using 2000 permutations. Using the SPAGeDi results, either F_{ST} or R_{ST} values were calculated between populations. F_{ST} was determined using Arlequin version 2.000 (Schneider *et al.* 2000) and R_{ST} was determined using RST Calc (Goodman 1997). Significance of both estimates was calculated using 10000 permutations in the respective programmes. Critical significance levels were corrected using Bonferroni tests (Rice 1989).

Neutral genetic variation in natural populations is dependent on population size (particularly effective population size) as well as on the amount of gene flow between populations (Hartl 1988; Frankham 1996; Vitalis & Couvet 2001). Isolated populations or small populations are likely to experience genetic drift resulting in the association of gene copies across loci (Hill & Robertson 1968). In order to

estimate population substructuring, gene identity was measured in ESTIM version 1.2, using a method-of-moments approach (Vitalis & Couvet 2001). Gene-identity (*F*) can be used as a measure of within-population genetic drift (Bray *et al*. 2009).

Significance of population genetic differentiation was performed using Fisher's exact test (Raymond & Rousset 1995) in GENEPOP, with the Markov chain set to 200 batches, 1000 iterations and 1000 dememorisations. Differences in both allelic and genotypic frequencies were tested for population pairs at each locus. Critical significance levels were corrected using Bonferroni tests (Rice 1989). An analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was used to determine the amount of genetic differentiation over all populations. Analyses were carried out using Arlequin version 2.000 (Schneider *et al.* 2000), with significance determined using 100000 permutations.

In order to explore relationships between individual genotypes, without *a priori* assumptions of grouping, a three dimensional factorial correspondence analysis (FCA) was carried out using GENETIX version 4.05.2 (Belkhir *et al*. 2004). The genotypic data are transformed into a contingency table (samples x alleles), where each individual genotype is represented by its score for alleles at different loci, i.e. 0 for absence, 1 for the allele in the heterozygous state and 2 for the homozygous state (She *et al*. 1987; Belkhir *et al*. 2004). Each individual is thus seen as a point in hyperspace, and relationships can be visualised (Belkhir *et al*. 2004).

Population assignment of individuals was performed using STRUCTURE version 2.1 (Pritchard *et al*. 2000). This model uses a Bayesian approach to assign individuals to *K* (unknown) populations. Individuals are assigned probabilistically to populations, or jointly to more than one population when they are genetically admixed. The admixture model was used with correlated allele frequencies. In order to determine *K*, 20 independent runs were performed for *K* values between one and 10 with a burn-in of 500000 and subsequent sampling of 1000000 iterations. From these runs, the true number of clusters (*K*) was determined using the "log probability of data" (Pr (X|*K*) approach, Pritchard *et al*. 2000) and the Δ*K* approach (Evanno *et al*. 2005). The 'Pr (X|*K*)' approach uses the posterior probability of *K* to determine the number of clusters, with values larger than the appropriate value of *K* resulting in a plateau and an increase in among-run variance (Pritchard *et al*. 2000; Pritchard & Wen 2004). The Δ*K* approach takes into consideration the variation around estimates of *K*, with the mode of the Δ*K* distribution providing a more likely estimate of the real number of clusters (Evanno *et al.* 2005). An assignment probability of $Q > 0.80$ (Lecis *et al.* 2006) was used as a threshold for assigning individuals to a specific cluster.

MtDNA amplification and sequencing

Samples from areas for which there is currently no mtDNA control region information were selected for amplification of the mtDNA control region. These samples included five elephants from Madikwe and one from PNP. A single elephant from AENP was selected to add to currently known data.

Approximately 500 bp of the 5' end of the mtDNA control region was amplified by using primers Laf CR1 (5'- GTA TAA GAC ATT ACA ATG GTC - 3') located in the $tRNA^{Pro}$ gene, and Laf CR2 (5'- AGA TGT CTT ATT TAA GAG GA - 3') located in the first conserved sequence block of the control region. These primers have been developed specifically to amplify this region of mtDNA in elephants (Nyakaana & Arctander 1999). Amplifications were carried out in 50 µl reaction volumes containing approximately 80 ng of extracted DNA, 2.5 mM $MgCl_2$, 1 x buffer, 50 pmol of each primer, 1 unit of Super-Therm *Taq* DNA polymerase (Southern Cross Biotechnology), and 2 mM dNTPs (Promega). The final volume was made up using SABAX® water. Amplification conditions were 94°C for 5 min, 35 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 1 min and final extension at 72°C for 5 min. All amplifications were carried out using a GeneAmp 9700 thermal cycler (Applied Biosystems). Electrophoresis of each PCR product was performed using 1% agarose gels (Laboratory Specialist Services) to determine whether amplification was successful. Negative controls were included during PCR and visualised on a gel to ensure that no cross-contamination of samples or stock solutions occurred during the setting up of PCR reactions. PCR products which amplified successfully were purified using the High Pure PCR Product Purification columns (Boehringer Mannheim) according to the manufacturer's protocol. Purified PCR products were sequenced in both directions by the dideoxy chain-termination method (Sanger *et al.* 1977). Approximately 120 ng of PCR products were cycle sequenced in a final volume of 10 µl with 3.2 pmol of primer (either Laf CR1 or Laf CR2) and 2 µl ABI PRISM Big Dye™ Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The final volume was made up using SABAX® water. The standard protocol (25 cycles of: 96°C for 10 s, 50°C for 5 s and 60°C for 4 min) for cycle sequencing was followed (Perkin Elmer 1995). Products were precipitated according to the standard sodium acetate precipitation protocol as described by Perkin Elmer (1995). The PCR products were sequenced on an ABI 377 automated sequencer (Applied Biosystems). Raw sequence data were evaluated using the Sequence Analysis® program (Applied Biosystems). Sequences from both the light and heavy strands of the mtDNA control region, in an individual, were compared to each other and a consensus sequence was produced for each animal in Sequence Navigator® (Applied Biosystems).

To further investigate relationships between populations in southern and eastern Africa, mtDNA control region sequences were obtained from various studies. GenBank sequences from South Africa,

Namibia, Botswana, Zimbabwe, Uganda, Kenya and Tanzania were included (Appendix 8). Sequences were obtained from a previous study on southern African elephants (R.J. van Aarde, Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria & T.J. Robinson, Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication); these included five sequences from Maputo Elephant Reserve (Mozambique), five sequences from Tembe Elephant Park (South Africa) and ten sequences from KNP (South Africa) (See Table 1, Figure 1).

MtDNA sequence analysis

Consensus sequences were aligned using ClustalX (Thompson *et al*. 1997) and were checked manually. Since the mtDNA control region is known to have a high mutation rate, homoplasy is likely to be observed in this region (Avise & Lansman 1983; Sanderson & Donoghue 1989; Galtier *et al*. 2006). Homoplasy is the independent evolution of the same character state (Lankester 1870) and results in haplotype networks that do not reflect the actual mutational distances between haplotypes (Avise & Lansman 1983; Goloboff 1991; Wake 1991). Homoplasic sites were identified by mapping all variable sites onto a minimum-spanning-network. These homoplasic characters were removed for further analyses.

In order to determine whether sampling localities could be combined for further analyses, allele frequency homogeneity tests among samples were performed using RandoChi, developed by W Delport (unpublished). This program uses the randomisation procedure for small sample sizes developed by Roff & Bentzen (1989). In this procedure the distribution of expected χ^2 values are generated using a Monte Carlo technique under the assumption that the null hypothesis of homogeneity cannot be rejected for this data set. By using this method, no assumptions about the underlying distributions are made, no lumping of data are required, and the accuracy of the estimate of α (the probability of rejecting a true null hypothesis) only depends on the number of randomisations of the original data set. A total of 10000 randomisations were performed.

Haplotypes were determined by calculating pairwise genetic distances using PAUP* version 4.0b10 (Phylogenetic Analysis Using Parsimony, Swofford 2003). Nucleotide diversity, π (the average number of nucleotide differences per site between two sequences, Nei 1987) and haplotype diversity (the probability that two randomly chosen mtDNA sequences are different in the sample, Nei 1987) were used to estimate genetic variation at three scales: (1) combined data set, (2) between regions (southern and eastern Africa) and (3) within each population. These diversity indices were estimated

using DnaSP version 3.51 (Rozas & Rozas 1999). Relationships between haplotypes were analysed by a minimum-spanning-network using MINSPNET (Excoffier & Smouse 1994).

An AMOVA (Excoffier *et al.* 1992) was used to determine the amount of genetic differentiation, firstly between the eastern and southern geographical regions and then between countries. This method is similar to other methods for calculating variance of gene frequencies, but takes into account the number of mutations between haplotypes. Analyses were carried out using Arlequin version 2.000 (Schneider *et al.* 2000), with significance determined using 100000 permutations.

Results

DNA Extraction

DNA was successfully extracted from all samples. Extracts did not show any DNA degradation and DNA concentrations ranged between 88 and 845 ng/µl for the tissue samples, between 7 and 20 ng/µl for the blood samples and between 4 and 144 ng/µl for the hair samples.

Microsatellite data analysis

Results from allele frequency homogeneity tests among sampling sites allowed for the lumping of Pilanesberg National Park, Phinda Private Game Reserve and Zimbabwe into the Pilanesberg group (Pilanesberg & Phinda χ^2 *p*-value = 0.251, $\alpha = 0.04$; Pilanesberg & Zimbabwe χ^2 *p*-value = 0.242, $\alpha = 0.02$; Phinda & Zimbabwe χ^2 *p*-value = 0.095, $\alpha = 0.02$), and Botswana and Zambia into the BotZam group (χ^2 *p*-value = 0.226, $\alpha = 0.03$). Sampling localities not placed into the above-mentioned groups were analysed as separate populations in further analyses.

After Bonferroni correction, linkage disequilibrium was only found between loci LA5 and LafMS03 in the KNP population ($p = 0.0053$). No loci were excluded from further analyses. The Hardy Weinberg global test showed significant heterozygote deficit at loci LA6, LafMS02 and LafMS05. These loci were found to have null alleles (Table 4). Loci LA5 and FH67, which did not show significant heterozygote deficit, were found to have null alleles (Null allele frequencies: LA5 = 0.14, FH67 = 0.08). A simulation study by Dakin & Avise (2004) found that null alleles with frequencies less than 0.2 would only result in slight underestimates of test statistics, therefore no loci were excluded from further analyses. Several loci in several populations deviated significantly from Hardy-Weinberg equilibrium (HWE), all of which were due to heterozygote deficit (positive F_{IS} values, Table 5). Although the heterozygote deficit observed may be due to a breakdown in elephant social behaviour (as seen in the Queen Elizabeth National Park population, Nyakaana *et al*. 2001), it is more likely that

we are seeing the effects of null alleles (Callen *et al*. 1993, Dakin & Avise 2004, Kalinowski 2006) or the effects of small sample sizes (van Treuren 1998, Vidya *et al*. 2005b, Lancaster *et al*. 2006). A test of conformance to HWE using a uniform sample size (six randomly selected individuals per group) indicated that only LA6 showed significant heterozygote deficit over the global tests (p = 0.004, results not shown). No deviations from HWE were observed due to heterozygote excess.

Table 4 Loci not in Hardy Weinberg Equilibrium (HWE) showing significant heterozygote deficit as well as null alleles in the global test. Bonferroni corrected rejection level ≤ 0.006. Probabilities for departure from HWE are given under '*p*-value'. F_{IS} values are given under 'F_{IS}'. The null allele frequencies (van Oosterhout estimate) are given under 'Null frequency'.

Locus	*p*-value	F_{IS}	Null frequency
LA6	0.000	0.26	0.19
LafMS02	0.000	0.29	0.14
LafMS05	0.002	0.35	0.08

Table 5 Sampling regions and loci not in Hardy-Weinberg Equilibrium (HWE) showing significant heterozygote deficit. Bonferroni corrected rejection level ≤ 0.006. Probabilities for departure from HWE are given under '*p*-value'. F_{IS} values are given under 'F_{IS}'.

Group	Locus	*p*-value	F_{IS}
Kruger	LafMS05	0.000	1.00
	FH67	0.005	0.21
Addo	LafMS02	0.001	0.32
Pilanesberg Group[1]	LafMS02	0.000	0.46
	FH39	0.004	0.12
Madikwe	LA6	0.001	0.41
	LafMS02	0.000	0.44

[1]Pilanesberg Group = Pilanesberg National Park, Phinda Private Game Reserve and samples from Zimbabwe

All the loci were highly polymorphic, with the most variable locus, FH39, having 12 alleles and the least variable loci, LafMS04 and LafMS05, each having four alleles across all groups. A total of 64 alleles were scored across all loci and populations. Genetic diversity statistics are summarised in Table 6. Expected heterozygosity varied from 0.27 to 0.68 and observed heterozygosity varied from 0.27 to 0.55. The AENP population consistently had the lowest expected and observed heterozygosities. The mean number of alleles per locus varied from 2.22 (AENP population) to 6.11 (Pilanesberg group). The mean number of alleles per polymorphic locus varied from 2.83 (AENP) to 6.11 (Pilanesberg group). Allelic frequencies per locus are summarised in Appendix 9. A total of nine private alleles were found in five sampling regions (Table 6). Locus LafMS05 was fixed for allele 154 in three sampling regions (AENP, Namibia and BotZam group) and the AENP population had three monomorphic loci (LA5, LA6, LafMS05).

Table 6 Mean expected heterozygosity (Exp Het), observed heterozygosity (Obs het), mean number of alleles per locus (Alleles/locus), mean number of alleles per polymorphic locus (Alleles/poly. locus) and the number of private alleles (Private alleles) observed for each of the elephant groups studied.

Group	Sample size	Exp Het	Obs Het	Alleles/locus	Alleles/poly. locus	Private alleles
Kruger	27	0.50	0.47	4.56	4.56	2
Addo	21	0.27	0.27	2.22	2.83	0
Pilanesberg Group[1]	48	0.54	0.47	6.11	6.11	1
Hluhluwe-iMfolozi	20	0.57	0.55	4.89	4.89	3
Madikwe	29	0.55	0.51	4.89	4.89	2
Namibia	6	0.68	0.55	3.33	3.63	1
BotZam Group[2]	15	0.51	0.47	3.56	3.88	0

[1]Pilansberg Group = Pilanesberg National Park, Phinda Private Game Reserve and samples from Zimbabwe.

[2]BotZam Group = Samples from Botswana and Zambia.

After Bonferroni correction, global multilocus and individual locus R_{ST} values were not significantly higher than pR_{ST} values (Table 7). Only one of 210 pairwise population comparisons had R_{ST} values significantly higher than pR_{ST} (HiP and Namibia, $p = 0.003$). For this data set, therefore, population differentiation is not strongly affected by allele size or stepwise mutation and thus F_{ST} is a more adequate estimate than R_{ST} (Hardy *et al.* 2003). Pairwise F_{ST} values ranged from 0.024 (KNP and Pilanesberg group) to 0.401 (KNP and AENP, Table 8). Only four of 21 (19%) pairwise F_{ST} comparisons were not significant after Bonferroni correction ($p \leq 0.006$). F_{ST} values indicate that the AENP population is significantly differentiated (values > 0.260) from all the other sampling regions. Gene identity probabilities (*F*) were low (-0.18 to 0.10; Namibia and KNP respectively), except for the AENP population which had a high value (0.52, Table 9).

Table 7 Mean multilocus and single locus F_{ST}, R_{ST} and pR_{ST} values of genetic differentiation as calculated in SPAGeDi (Hardy & Vekemans 2002). Comparisons (observed > expected) after Bonferroni correction were not significant.

Locus	**F_{ST}**	**R_{ST}**	***p*R_{ST} (95% CI)**
All loci	0.105	0.162	0.113 (0.049 to 0.195)
FH19	0.064	0.048	0.060 (-0.001 to 0.170)
FH39	0.126	0.322	0.118 (-0.003 to 0.310)
LA5	0.237	0.013	0.239 (0.053 to 0.521)
LA6	0.238	0.246	0.216 (0.089 to 0.295)
LafMS02	0.020	0.009	0.021 (-0.029 to 0.113)
LafMS03	0.051	0.145	0.074 (-0.006 to 0.207)
LafMS04	0.015	0.035	0.016 (-0.005 to 0.036)
LafMS05	0.002	-0.004	0.003 (-0.025 to 0.037)
FH67	0.068	0.089	0.057 (-0.017 to 0.125)

Table 8 Pairwise F_{ST} values for elephant sampling regions based on nine microsatellite loci. Bonferroni corrected significant values ≤ 0.006 indicated with *.

	Pil	HUP	Mad	KNP	Nam	Bot
Addo	0.342*	0.388*	0.260*	0.401*	0.355*	0.275*
Pil		0.098*	0.045*	0.024*	0.074	0.032
HUP			0.119*	0.117*	0.062	0.112*
Mad				0.060*	0.063*	0.041*
KNP					0.107*	0.048*
Nam						0.062

Group names: Addo – Addo Elephant National Park, Pil - Pilanesberg Group[1], HUP - Hluhluwe-iMfolozi Park, Mad – Madikwe Game Reserve, KNP – Kruger National Park, Nam – Etosha National Park, Namibia, Bot - BotZam Group[1].

[1]Pilanesberg Group and BotZam Group as in Table 6.

Table 9 Estimation of averaged one-locus identity probabilities (*F*) for each sampling group as calculated in ESTIM version 1.2 (Vitalis & Couvet 2001).

Group	*F*
Addo	0.52
Kruger	0.10
BotZam Group[1]	0.09
Pilanesberg Group[1]	0.04
Madikwe	0.02
Hluhluwe-iMfolozi	-0.01
Namibia	-0.18

[1]Pilanesberg Group and BotZam Group as in Table 6.

Exact tests for population differentiation based on allelic and genotypic frequencies showed all population groupings to be significantly differentiated from each other (results not shown). AMOVA analysis, however, showed that 85.66% of the variance was within populations (Φ_{ST} = 0.143, p < 0.01).

The FCA for all individuals clearly showed that AENP individuals cluster together, with overlap between other regions (Figure 2). The first three axes cumulatively explained 15.1% of the observed genetic diversity. The exclusion of AENP individuals showed the slight separation of HiP individuals, with the first three axes explaining 14.53% of the genetic diversity (Figure 3). No further clusters were visible between individuals from the other sampling regions when AENP and HiP individuals were excluded from the analysis (results not shown).

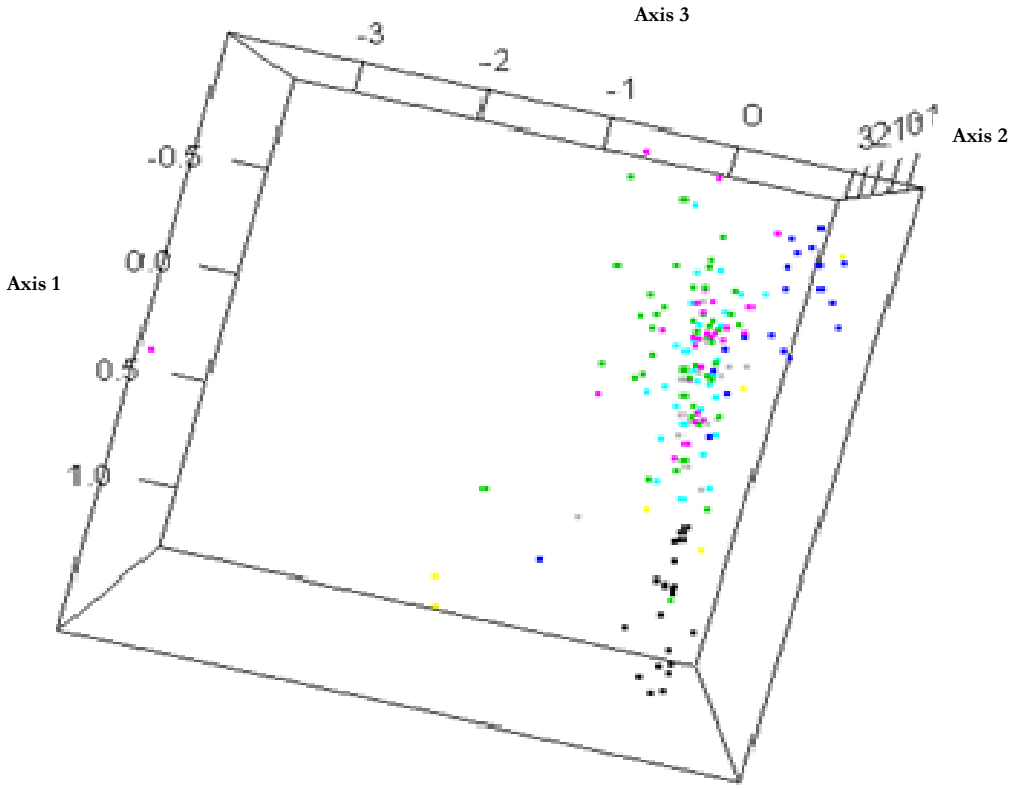

Figure 2 Three-dimensional factorial correspondence analysis using allele frequencies for the full data set. Axis 1 – 5.54%, Axis 2 – 5.12%, Axis 3 – 4.44%, Cumulative – 15.10%. Key: Black – Addo Elephant National Park, Green - Pilanesberg Group (including Pilanesberg National Park, Phinda Private Game Reserve and Zimbabwe individuals), Dark blue – Hluhluwe-iMfolozi Park, Aquamarine – Madikwe Game Reserve, Purple – Kruger National Park, Yellow – Etosha National Park, Namibia, Grey - BotZam Group (including Botswana and Zambian individuals).

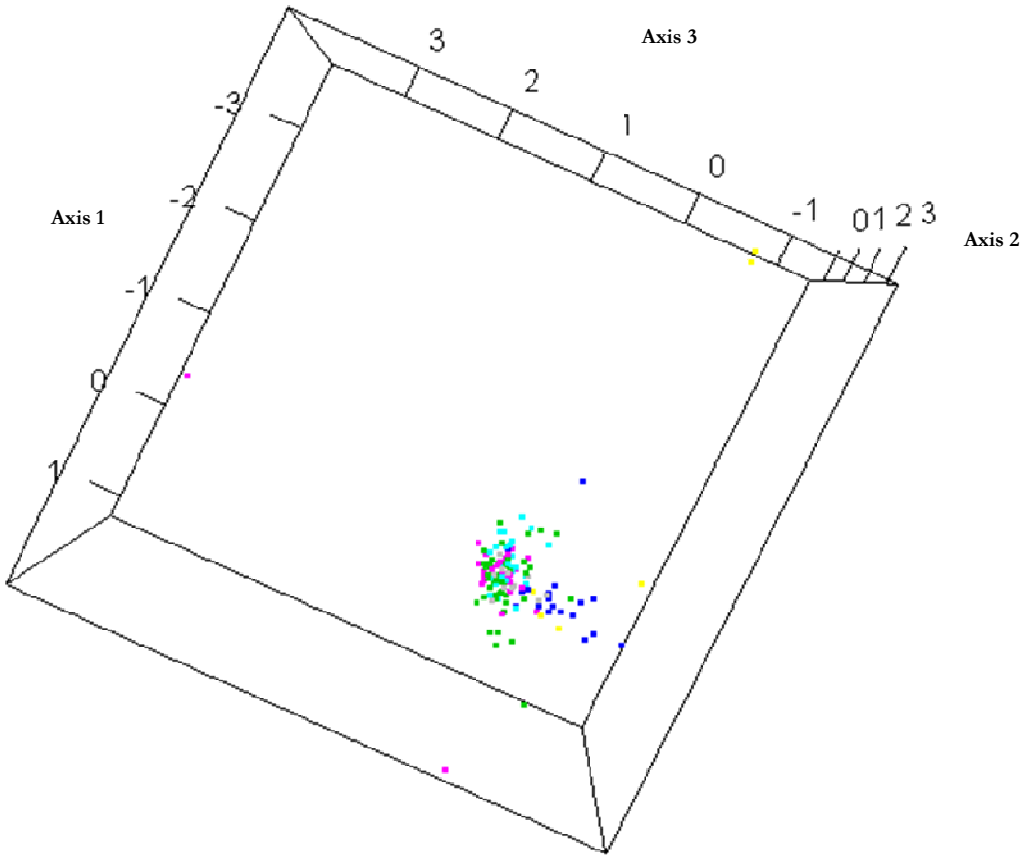

Figure 3 Three dimensional factorial correspondence analysis using allele frequencies for the data set excluding Addo Elephant National Park individuals. Axis 1 – 5.44%, Axis 2 – 4.69%, Axis 3 – 4.40%, Cumulative – 14.53%. Key: Green – Pilanesberg Group (including Pilanesberg National Park, Phinda private Game Reserve and Zimbabwe individuals), Dark blue – Hluhluwe-iMfolozi Park, Aquamarine – Madikwe Game Reserve, Purple – Kruger National Park, Yellow – Etosha National Park, Namibia, Grey – BotZam Group (including Botswana and Zambian individuals).

The number of clusters for STRUCTURE could not be clearly identified using either the 'Pr (X/*K*)' approach or the Δ*K* approach. Ideally, in the 'Pr (X/*K*)' approach values larger than the appropriate value of *K* result in the probability of *K* showing a plateau (Pritchard & Wen 2004), but our results did not indicate a plateau for any value of *K*, however from *K* = 5 the standard deviation was significantly increased (Figure 4A). Despite a distinct peak at *K* = 6 using the Δ*K* approach (Figure 4B), interpreting both methods of determining *K* makes it impossible to exclude any value of *K* from one to four. Cluster results, averaged over five runs, show AENP individuals repeatedly being assigned to one cluster for varying values of *K*, with Q values (the probability of individual assignment) greater than 0.80 (Figure 5). Individuals, excluding AENP individuals, were assigned "roughly symmetrically" to each population when *K* = 3 and *K* = 4 indicating no real population structure for these values of *K* (Pritchard & Wen 2004), therefore it is more likely that *K* = 2. A single individual from the Pilanesberg group was repeatedly assigned to the AENP cluster across all values of *K* (Q = 0.89 when *K* = 4).

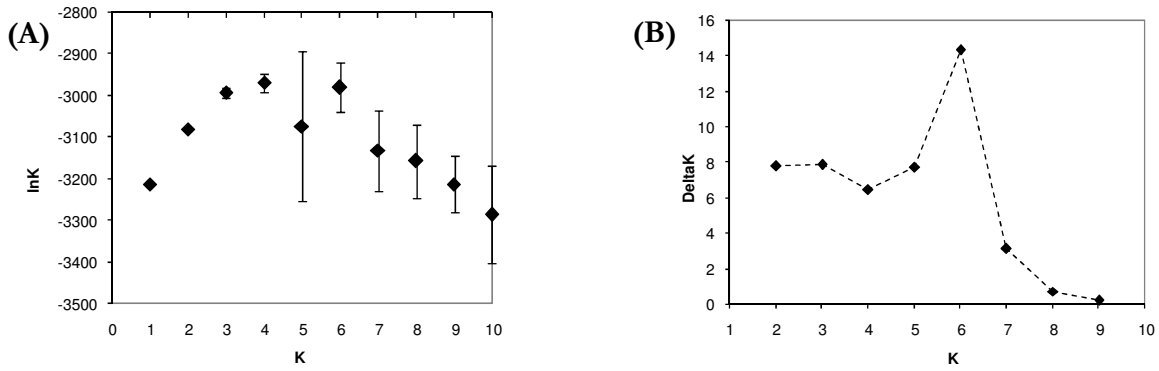

Figure 4 Determination of the ideal number of clusters (*K*) for use in STRUCTURE using (**A**) the posterior probability of *K*, LnK, averaged across 20 runs for values of *K* between one and 10 (bars indicate the standard deviations across 20 iterations), and **(B)** Δ*K* (a measure of the rate of change in the STRUCTURE likelihood function) for values of *K* between two and nine.

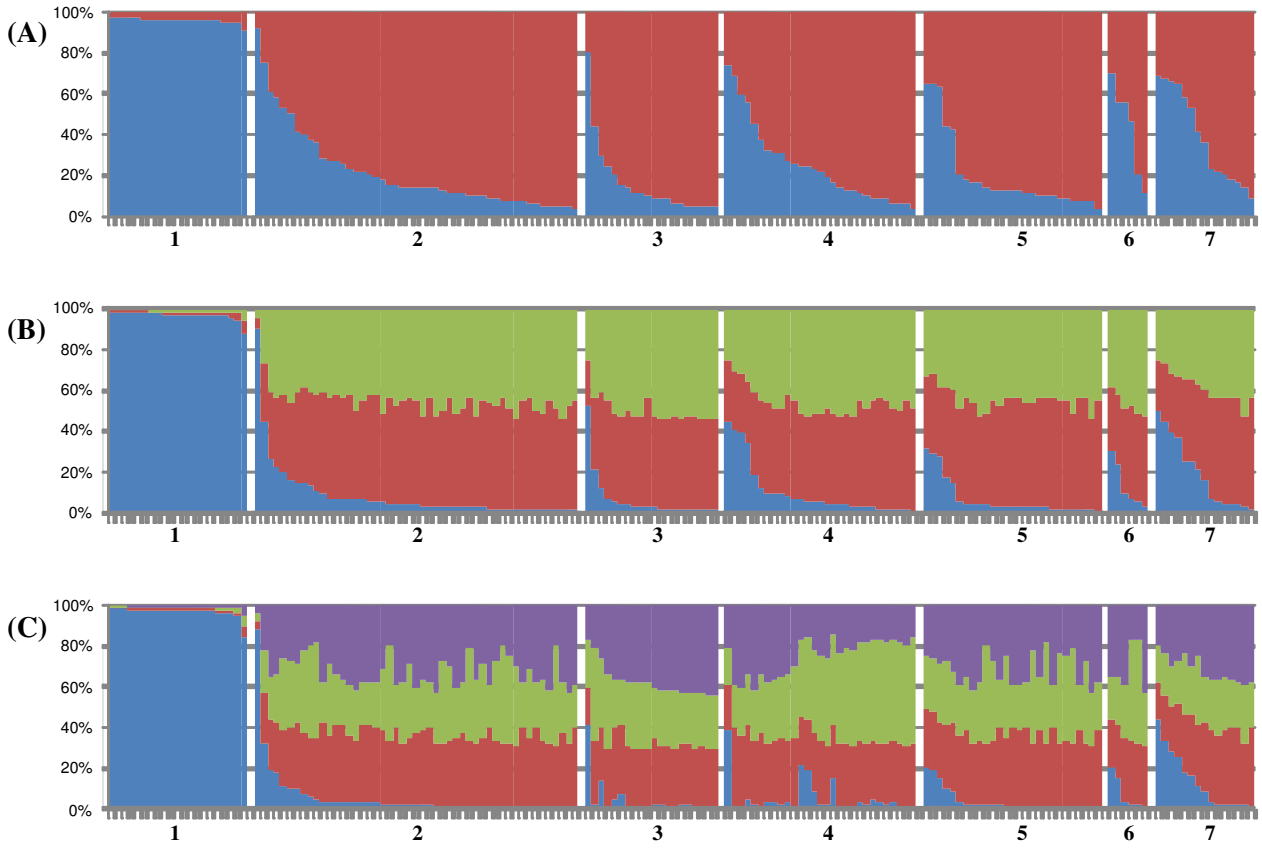

Figure 5 Structure for **(A)** $K = 2$, **(B)** $K = 3$ and **(C)** $K = 4$ using the full data set, averaged over five runs. Each individual is represented by a thin vertical line, which is partitioned into various coloured segments representing the individual's estimated membership fractions (indicated on left axis). Vertical white lines separate individuals from various groups. Groups: 1 – Addo Elephant National Park, 2 – Pilanesberg Group (including Pilanesberg National Park, Phinda Private Game Reserve and Zimbabwe individuals), 3 – Hluhluwe-iMfolozi Park, 4 – Madikwe Game Reserve, 5 – Kruger National Park, 6 – Etosha National Park, Namibia, 7 – BotZam Group (including Botswana and Zambian individuals).

A further STRUCTURE analysis was carried out excluding AENP individuals. Although the Δ*K* approach showed a peak at *K* = 3, we could still not exclude *K* values less than three (Figure 6A & B). Cluster results showed no real population structure for *K* = 2 and *K* = 3 (Figure 7A & B).

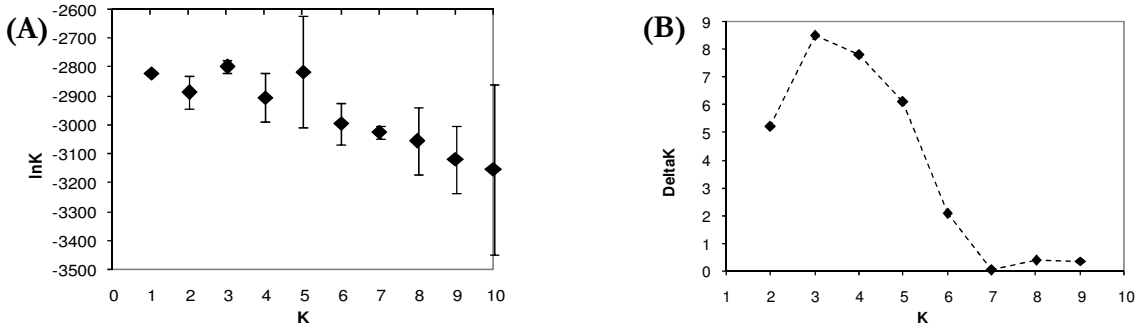

Figure 6 Determination of the ideal number of clusters (*K*) for use in STRUCTURE, with Addo Elephant National Park individuals excluded, using **(A)** the posterior probability of *K*, LnK, averaged across 20 runs for values of *K* between one and 10 (bars indicate the standard deviations across 20 iterations), and **(B)** Δ*K* (a measure of the rate of change in the STRUCTURE likelihood function) for values of *K* between two and nine.

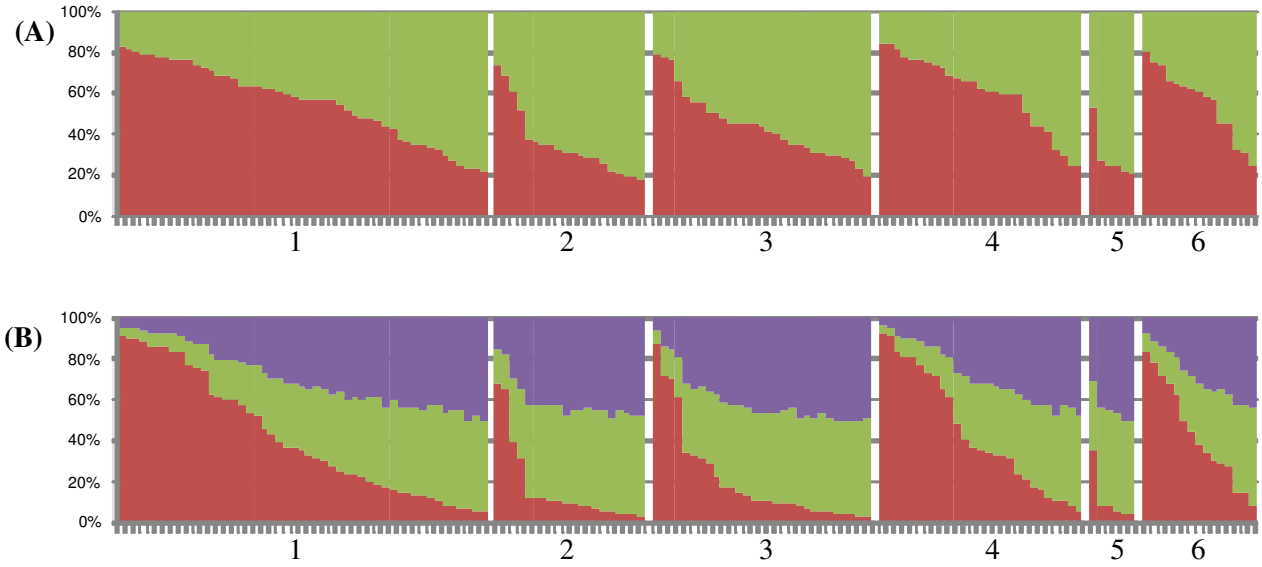

Figure 7 Structure for **(A)** *K* = 2 and **(B)** *K* = 3, excluding Addo Elephant National Park individuals, averaged over five runs. Each individual is represented by a thin vertical line, which is partitioned into various coloured segments representing the individual's estimated membership fraction (indicated on left axis). Vertical white lines separate individuals from various groups. Groups: 1 – Pilanesberg Group (including Pilanesberg National Park, Phinda Private Game Reserve and Zimbabwe individuals), 2 – Hluhluwe-iMfolozi Park, 3 – Madikwe Game Reserve, 4 – Kruger national Park, 5 – Etosha National Park, Namibia, 6 – BotZam Group (including Botswana and Zambian individuals).

MtDNA sequence analysis

Seven sequences of approximately 600 bp were generated from the samples selected. For the combined data set, including individuals from southern and eastern Africa, only 430 bp could be used for comparison, of which 19 sites showed homoplasy and were removed. Further analyses were based on 411 bp, of which 25 sites (6.1%) were variable (Table 10) defining 22 haplotypes, all of which have been identified in previous studies (Nyakaana & Arctander 1999; Eggert *et al*. 2002; Nyakaana *et al*. 2002). Of these variable sites one involved both a transitional and transversional substitution, one was a transversional substitution and 23 sites were transitional substitutions. Nine haplotypes were represented by more than one individual (Table 11), two were restricted to southern African populations (664KNP, ZBE2), four were restricted to eastern African populations (QE4, KV1, KV&, KV8) and three had individuals from both southern and eastern African populations (649MOC, 647MOC, QE13). Most of the singleton haplotypes were found in the southern African region.

Sampling localities were combined taking into consideration results from allele frequency homogeneity tests, the distance between populations and translocation history. This allowed the lumping of Kruger National Park, Pilanesberg National Park, Madikwe Game Reserve and Tembe Elephant Reserve into the Kruger group ($\chi 2 = 4.729$, p-value = 0.339, Pilanesberg and Madikwe both have founders from Kruger; Kruger and Tembe are <300km apart), and Namibia and Botswana into the Namibia + Botswana group ($\chi^2 = 3.822$, p-value = 0.095, <400km apart). All other sampling localities were kept as separate populations for further analyses.

Nucleotide diversity in the combined data set was 0.8%. For southern Africa the nucleotide diversity was 0.43% and for eastern Africa 1.44%. Nucleotide diversity ranged widely between populations from 0.08% for the Kruger group to 1.36% for the Amboseli National Park population (Table 12). Haplotype diversity in the combined data set was 0.76. For southern Africa the haplotype diversity was 0.56 and for eastern Africa 0.97. Haplotype diversities varied from 0.16 to 1.00 (Table 12).

The minimum-spanning-network showed no real support for the two savanna haplogroups identified in previous studies (Eggert *et al*. 2002; Nyakaana *et al*. 2002; Johnson *et al*. 2007), with no more than two mutational steps separating haplotypes (Figure 8). AMOVA analysis showed that on a continental level 77% of the genetic variation was within populations ($\Phi_{ST} = 0.228$, $p = 0.04$) (Table 13). The grouping of populations into regional groups and country groups revealed that most of the genetic variation, although lower than on a continental level, was within populations. Differentiation among populations within groups, although much lower than within populations, was found to be significant for the regional and country groups.

Table 10 Variable sites observed in a 411 bp fragment of the 22 mitochondrial DNA control region haplotypes identified from 72 sequences analysed. Haplotypes are given on the left. The vertical numbers indicate the positions of the polymorphic sites relative to haplotype 664KNP.

	Polymorphic sites																								
	34	58	63	64	82	97	102	132	143	155	211	213	217	220	228	242	245	251	252	253	266	269	303	304	362
Haplotypes																									
664KNP	A	G	-	T	C	T	C	A	C	T	T	A	G	A	T	A	C	T	G	T	A	A	C	T	T
KG1	.	.	-	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
647Moc	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
649Moc	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.
ZBE1	.	A	A	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ZBE2	.	A	A	C	.	C	.	.	.	C	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.
ZBE3	.	.	G	.	T	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ZBE5	.	A	-	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
BOT6	G	A	A	C	.	.	.	.	.	C	C	.	.	.	C	.	.	.	.	.	.	.	.	.	.
WC6	.	A	A	C	.	.	T	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KH2	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
KV1	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
KV7	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	C	.
KV8	G	A	A	C	.	.	.	.	.	C	C	.	A	.	C	.	.	.	.	.	.	.	.	.	.
KV28	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.
QE4	G	A	A	C	.	.	.	.	.	C	C	.	A	.	C	G	.	.	.	.	.	.	.	.	.
QE13	.	A	A	C	.	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MM20	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.
AM1	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.
AM2	.	A	-	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	C	.	.	.	.	.
SA8	.	A	-	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	C	.	.	.	C	.
AF8	G	A	A	C	.	.	.	.	.	C	C	.	A	G	C	G	.	.	.	.	.	.	.	.	G

A dot (.) indicates identity with haplotype 664KNP, a dash (-) indicates an inferred insertion/deletion event and a question mark (?) indicates missing data.

Haplotypes: 664KNP – Kruger National Park, South Africa; 647Moc and 649Moc – Mozambique (R.J. van Aarde, Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria & T.J. Robinson, Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication); for other haplotype references see Appendix 8.

Table 11 Haplotypes identified, from the combined data set, represented by more than one individual.

Haplotype	Sequences	Origin
QE4	QE4	Queen Elizabeth National Park, Uganda
	AF8	Kenya, currently in North American Zoo
	AF10	Kenya, currently in North American Zoo
649Moc	648Moc	Mozambique
	649Moc	Mozambique
	651Moc	Mozambique
	653Tem	Tembe Elephant Park, South Africa
	KV2	Kidepo Valley National Park, Uganda
	AM12	Amboseli National Park, Kenya
647Moc	647Moc	Mozambique
	669KNP	Kruger National Park, South Africa
	670KNP	Kruger National Park, South Africa
	671KNP	Kruger National Park, South Africa
	Addo5	Addo Elephant National Park, South Africa
	QE1	Queen Elizabeth National Park, Uganda
	AF219239	Tanzania
	BOT4	Chobe National Park, Botswana
	WC2	Western Caprivi Recreation Park, Namibia
	WC4	Western Caprivi Recreation Park, Namibia
	WC13	Western Caprivi Recreation Park, Namibia
664KNP	LMT1	Madikwe Game Reserve, South Africa
	LMT2	Madikwe Game Reserve, South Africa
	LMT4	Madikwe Game Reserve, South Africa
	LMT5	Madikwe Game Reserve, South Africa
	Addo1	Addo Elephant National Park, South Africa
	p6229	Addo Elephant National Park, South Africa
	p6230	Pilanesberg National Park, South Africa
	p7350	Madikwe Game Reserve, South Africa
	663KNP	Kruger National Park, South Africa
	664KNP	Kruger National Park, South Africa
	665KNP	Kruger National Park, South Africa
	666KNP	Kruger National Park, South Africa
	667KNP	Kruger National Park, South Africa
	668KNP	Kruger National Park, South Africa
	672KNP	Kruger National Park, South Africa
	KG2	Kruger National Park, South Africa
	652Tem	Tembe Elephant Park, South Africa
	654Tem	Tembe Elephant Park, South Africa
	655Tem	Tembe Elephant Park, South Africa
	656Tem	Tembe Elephant Park, South Africa
	650Moc	Mozambique
	ZBE4	Zimbabwe

References for published mtDNA sequences can be found in Appendix 8.

Unpublished sequences: 'Moc', 'KNP', 'Tembe' – R.J. van Aarde (Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria, personal communication) & T.J Robinson (Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication), 'LMT', 'p' – this study.

Table 11 continued

Haplotype	Sequences	Origin
KV1	KV1	Kidepo Valley National Park, Uganda
	KV17	Kidepo Valley National Park, Uganda
	MM4	Masai Mara Game Reserve, Kenya
KV7	KV7	Kidepo Valley National Park, Uganda
	MM19	Masai Mara Game Reserve, Kenya
KV8	KV8	Kidepo Valley National Park, Uganda
	MF1	Murchinson Falls National Park, Uganda
	MF5	Murchinson Falls, National Park, Uganda
	AM10	Amboseli National Park, Kenya
ZBE2	ZBE2	Zimbabwe
	BOT9	Kwando, Botswana
QE13	QE13	Queen Elizabeth National Park, Uganda
	ZBE6	Zimbabwe
	BOT2	Chobe National Park, Botswana
	BOT15	Ngwasha, Botswana
	BOT16	Nunga Valley, Botswana
	BOT21	Sibuyu Forest Reserve, Botswana

References for published mtDNA sequences can be found in Appendix 8.

Unpublished sequences: 'Moc', 'KNP', 'Tembe' – R.J. van Aarde (Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria, personal communication) & T.J. Robinson (Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication), 'LMT', 'p' – this study.

Table 12 Summary statistics for mitochondrial DNA control region variation in 13 sampling regions. The group "Zoo Kenya" refers to sequences from Kenyan elephants currently in North American Zoos (see Eggert *et al*. 2002).

Sampling region	No. of sequences	No. of haplotypes	Nucleotide diversity (%)	Haplotype diversity
Kruger Group[1]	23	3	0.08	0.16
Addo Elephant NP	3	1	0.00	0.00
Mozambique	5	3	0.39	0.70
Namibia + Botswana	12	6	0.69	0.82
Zimbabwe	6	4	1.04	1.00
Masai Mara GR	3	3	0.35	1.00
Amboseli NP	4	4	1.36	1.00
Samburu NP	1	1	0.00	0.00
Kidepo Valley NP	6	5	0.97	0.93
Murchinson Falls NP	2	2	0.26	1.00
Queen Elizabeth NP	3	3	1.23	1.00
Zoo Kenya	3	2	0.26	1.00
Tanzania	1	1	0.00	0.00
TOTAL	72	22	0.80	0.76

NP = National Park, GR = Game Reserve.

[1]Kruger Group = Kruger National Park, Pilanesberg National Park, Madikwe Game Reserve and Tembe Elephant Park.

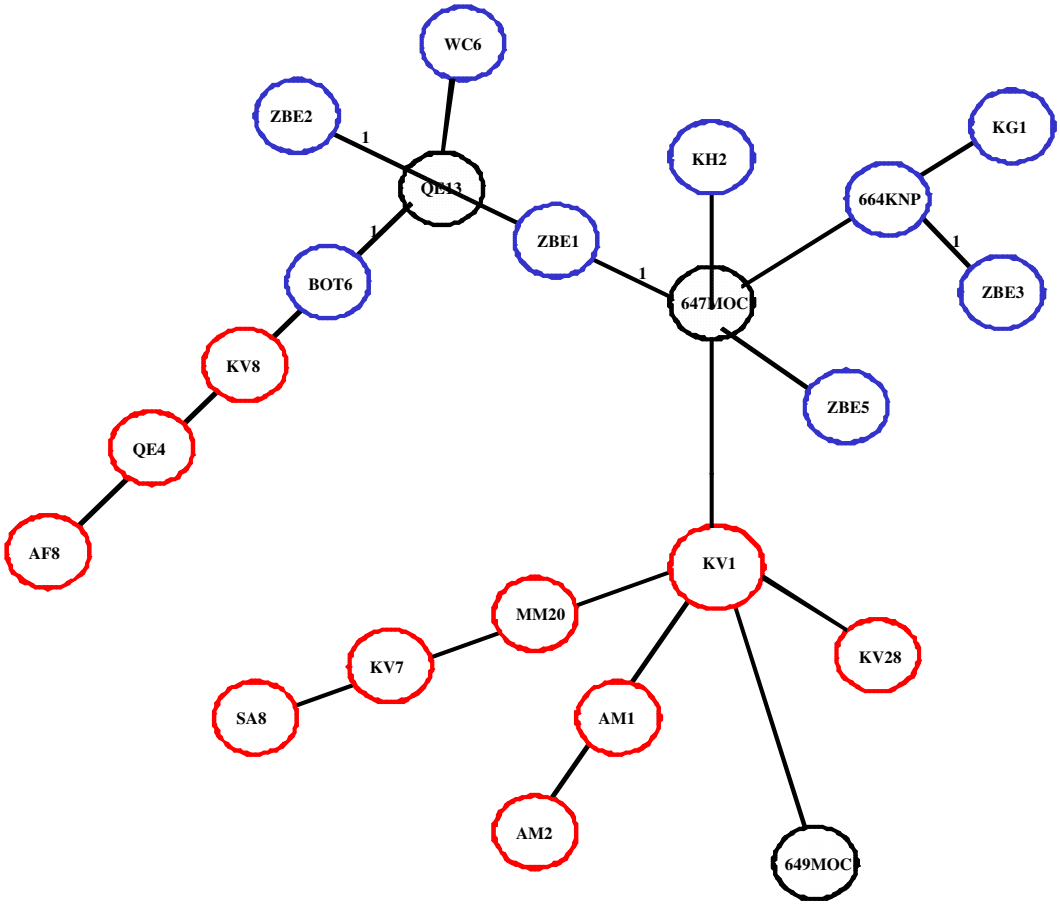

Figure 8 Minimum-spanning-network of the 22 mitochondrial DNA control region haplotypes identified. Red circles indicate haplotypes from eastern Africa and blue circles haplotypes from southern Africa. Shaded circles indicate that both southern and eastern African individuals are included. Lines between circles indicate one mutational step between haplotypes. Numbers next to connecting lines indicate mutational steps over and above the single mutation step linking the haplotypes. Haplotype key can be found in Table 10 and Appendix 8.

Table 13 Hierarchical analysis of molecular variance (AMOVA) for *a priori* defined groups based on mitochondrial DNA control region sequences.

Grouping	% Total variance			Φ Statistic		
	Among groups	Among populations within groups	Within populations	Φ_{CT}	Φ_{SC}	Φ_{ST}
Continental		22.82	77.18			0.228*
Southern/ Eastern Africa	4.56	19.95	75.48	0.046	0.209**	0.245*
Countries	7.25	15.95	76.80	0.073	0.172*	0.232*

* = $p < 0.05$, ** = $p < 0.01$

Discussion

This study presents a first assessment of the genetic diversity of reintroduced elephant populations in South Africa and compares them to the source population from the KNP, and a bottlenecked population in the AENP. Most genetic studies of elephants have focused on the continental level, with only three published studies looking at diversity of populations within countries, namely Uganda (Nyakaana & Arctander 1999), Kenya (Okello *et al*. 2008b), and South Africa (Whitehouse & Harley 2001). As Okello *et al*. (2008b) stated, studies at this level not only add to previous broad scale studies, but are crucial for providing information for future conservation management plans.

The reintroduced populations included in the Pilanesberg group (including PNP, Phinda and Zimbabwe), HiP, and Madikwe reflect similar heterozygosities to and allelic richness greater than the Kruger population, one of the source populations. Since our samples were a combination of founding individuals and their first generation offspring, the genetic diversity of these populations not only suggests a reduced extinction risk (Frankham 2005) but the ability to adapt to future environmental changes (Lacy 1997). Populations that undergo a reduction in size, like those that form the founders of a translocated population, are likely to experience a decrease in allelic diversity and heterozygosity due to stochastic events (Meffe & Carroll 1997; Frankham 2005). However, allelic diversity tends to decline faster than heterozygosity, since the rare alleles that are lost do not affect heterozygosity (Nei *et al*. 1975; Cornuet & Luikart 1996). A reduction in genetic diversity can lead to inbreeding which can potentially lead to inbreeding depression resulting in increased extinction risk. Examples where this has been observed include the Florida panther (*Felis concolor coryi*), greater prairie-chicken (*Tympanuchus cupido pinnatus*), and koala (*Phascolarctos cinereus*) (reviewed in Hedrick & Kalinowski 2000; Keller & Waller 2002; Frankham 2003; Frankham 2010a). Reduced genetic diversity may result in a reduced ability to cope with environmental changes (Frankham 2003). These environmental changes can include new diseases, pests, parasites, competitors and predators, pollution, climatic cycles and climatic change (Frankham *et al*. 2002). Examples where environmental changes have increased extinction risk include Soay sheep (*Ovis aries*, Coltman *et al*. 1999), and harbour seals (*Phoca vitulina*, Rijks *et al*. 2008). Therefore, the preservation of genetic diversity in reintroduced populations is crucial, and the monitoring of this genetic diversity will provide input for future management decisions (Schwartz *et al*. 2007).

The genetic diversity of the sampling localities studied here fall within the ranges observed for elephant populations in southern Africa and populations of Asian elephants (*Elephas maximus*) (see Appendix 10). Southern African populations have in previous and in the current study been found to

have a lower genetic diversity than those in eastern Africa (see Appendix 10). The difference in diversity may be attributable to the history of the two regions. Populations in eastern Africa are recovering from a more recent bottleneck, where individuals born prior to the population reductions (considering elephants live for approximately 60 years, Skinner & Chimimba 2005) may still form part of the effective population (the individuals involved in breeding in a population, Crow & Kimura 1970; Hartl 1988) (Okello *et al*. 2008b). Southern African populations, on the other hand, have been recovering for approximately 100 years (at least two elephant generations) and despite certain populations having recovered quickly, the reduction in genetic diversity across the region appears to have been more severe (Whitehouse & Harley 2001). The difference in long term population sizes, and consequently effective population size, between the two regions may have lead to the difference seen in genetic diversity (Crow & Kimura 1970; Frankham 1996; Schwartz *et al*. 1998; Montgomery *et al*. 2000). It would be interesting to see if a similar loss of genetic diversity will be found in the eastern African populations 100 years from now (Whitehouse & Harley 2001).

Based on F_{ST} values from microsatellite data, South African populations, excluding AENP, show low population differentiation. This can be explained by the recent translocations of individuals from KNP to PNP, HiP and Madikwe. STRUCTURE results give further support to a mixed gene pool within southern Africa. We would have expected HiP to have had similar F_{ST} values to that of PNP since both are wholly founded by Kruger individuals. PCA analysis also highlights HiP's difference to other populations. We may be observing initial signs of genetic drift, or the founding individuals from KNP may have had a different genetic diversity (HiP has three private alleles) to that observed in PNP founders and KNP individuals studied here. Namibia and Botswana had low differentiation, probably reflecting not only ongoing gene flow in southern Africa (Hall-Martin 1992), but insufficient time for genetic drift to have developed in these populations (Hedrick 1999).

Addo as an example of a bottlenecked population

Both this study and the Whitehouse and Harley (2001) study found that the AENP population had low levels of genetic diversity compared to any other elephant population. This has been attributed to the severe bottleneck this population experienced in the 1930's, when the population was reduced to only 11 elephants (Trollope 1931; Whitehouse & Harley 2001). Reduced levels of genetic diversity have been seen in other African mammals that are known to have undergone population bottlenecks, for example, roan antelope (*Hippotragus equines*, Alpers *et al*. 2004), black wildebeest (*Connochaetes gnou*, Alais 2000; Grobler *et al*. 2005), Cape mountain zebra (*Equus zebra zebra*, Moodley & Harley 2005), kob antelope (*Kobus kob*, Lorenzen *et al*. 2007), southern white rhino (*Ceratotherium simum simum*, Scott 2008), and walia ibex (*Capra walie*, Gebremedhin *et al*. 2009).

The reduction in size of the AENP population, and its concurrent isolation from the KNP population (Hall-Martin 1992) not only resulted in a loss of genetic diversity (Whitehouse & Harley 2001) but led to genetic drift. The effects of genetic drift can be clearly identified by AENP's differentiation from other populations as identified in F_{ST}, PCA and STRUCTURE analyses. The population's isolation history is also reflected in the ESTIM F statistic values, which are higher than those found in isolated and endangered cattle populations (Bray *et al*. 2009), and in highly inbred captive populations of goodeid fish (*Ameca splendens*, Bailey *et al*. 2007). Similar effects of genetic drift have been observed in greater prairie-chickens (*Tympanuchus cupido pinnatus*, Johnson *et al*. 2003), bridled nailtail wallabies (*Onychogalea fraenata*, Sigg 2006), and adders (*Vipera berus*, Ursenbacher *et al*. 2009).

Whitehouse and Harley (2001) suggested the introduction of elephants from elsewhere into the AENP population in order to boost the population's genetic diversity. Eight male elephants from the KNP were translocated to AENP in 2002 and 2003 (one died in 2004; K. Gough, Terrestrial Ecology Research Unit, Department of Zoology, University of Port Elizabeth, personal communication). Subsequently 11 AENP bulls were translocated to local private game reserves in 2005. The genetic success of the translocation from KNP still needs to be investigated (K. Gough, Terrestrial Ecology Research Unit, Department of Zoology, University of Port Elizabeth, personal communication).

MtDNA regional diversity

Nucleotide and haplotype diversities in the mtDNA control region are within the range observed in other African elephant studies (Appendix 11), although many of the haplotypes are from previous studies. The nucleotide diversity observed is much lower than that of many African mammals, for example, greater kudu (2.7%, *Tragelaphus strepsiceros*, Sakwa 2001), hartebeest (3.2%, *Alcelaphus buselaphus*, Arctander *et al*. 1999), water-buck (4.1%, *Kobus ellipsiprymnus*, Simonsen 1997), black rhino (4.3%, *Diceros bicornis minor*, Brown & Houlden 2000), and African buffalo (4.5%, *Syncerus caffer*, Heller *et al*. 2008), but are similar to the values found in Asian elephants (Appendix 11).

Two distinct haplogroups with broad geographical overlap have previously been identified from mtDNA data (Eggert *et al*. 2002; Nyakaana *et al*. 2002; Johnson *et al*. 2007). The addition of mtDNA control region sequences (obtained in this study and by R.J. van Aarde, Conservation Ecology Research Unit, Department of Zoology and Entomology, University of Pretoria, Pretoria & T.J. Robinson, Evolutionary Genomics Group, Department of Zoology, University of Stellenbosch, personal communication) to the published data set did not provide support for these previously identified haplogroups, but do show broad geographical overlap of haplotypes. Weak genetic structure at the mitochondrial level is further supported by AMOVA results, where the majority of genetic

variation was within populations. The inability to detect the two distinct haplogroups in this study may be related to the removal of homoplasic characters from further mtDNA sequence analyses, whereas there is no mention of the removal of homoplasic sites by previous studies (Eggert *et al*. 2002; Nyakaana *et al*. 2002; Johnson *et al*. 2007). Homoplasy is likely to be a problem in the control region of mtDNA due to its high mutation rate (Avise & Lansman 1983; Sanderson & Donoghue 1989; Galtier *et al*. 2006), and is believed to result in haplotype networks that do not reflect the actual mutational distances between haplotypes (Avise & Lansman 1983; Goloboff 1991; Wake 1991). Unfortunately it is difficult to determine which minimum-spanning-network is a true reflection of elephant history; future phylogeographic research should investigate the effect of homoplasy and include other loci, for example nuclear loci (Roca *et al*. 2001; Roca *et al*. 2005), and different mtDNA regions, for example ND5 or COI (Roca *et al*. 2005), to disentangle the history of 'savanna' elephants.

Genetic considerations for future management

Since elephants are large bodied, long-lived mammals with long generation times the genetic effects of small population size on the translocated populations are unlikely to be seen in our current lifetime. Fortunately the effects of reduced elephant population sizes can be observed in the AENP population, since this has been a relatively small and isolated population for approximately 150 years (Whitehouse 2001). There is a wide body of data showing the effects of reduced genetic diversity on small isolated populations, including genetic drift, inbreeding depression and a reduced ability to cope with environmental changes, all of which lead to increased extinction risk or extinction itself (reviewed in Hedrick & Kalinowski 2000; Keller & Waller 2002; Frankham *et al*. 2002; Frankham 2005; Frankham 2010a, b).

Therefore certain genetic considerations need to be kept in mind when managing these populations (Meffe & Carroll 1997; Frankham *et al*. 2002). These include:

1. The maintenance or improvement of genetic diversity in populations, i.e. founder populations should have the same or greater genetic diversity than the original population, so as to maintain future levels of diversity and allow for evolutionary potential (Lacy 1997; Frankham 2005). This includes having population sizes that are as large as possible. The larger a population is, the less likely it is to lose genetic diversity (Hartl 1988; Frankham 1996). Despite founding populations being small in number, high population growth rates (see Chapter 2) may prevent the loss of genetic diversity. However, population sizes will be constrained by reserve sizes.
2. The prevention of genetic drift in small, isolated populations by increasing gene flow between them (Frankham *et al*. 2002). Ideally gene flow between populations should be advocated, for example between KNP and PNP or HiP; see later discussion on management units. Behavioural problems

(Slotow & van Dyk 2001; Slotow *et al*. 2001; Whitehouse 2001) and the physical movement of individuals may make this difficult (Slotow *et al*. 2000), however the development of corridors between reserves may allow future exchange of genetic material (van Aarde *et al*. 2008)

3. Identification of founder effect, genetic drift, bottlenecks or inbreeding (Frankham *et al*. 2002). Future studies will need to monitor the genetic diversity of populations at various time scales; this will aid in determining whether all founders have successfully been involved in breeding or whether new genetic material needs to be introduced into the population (Schwartz *et al*. 2007). Inbreeding depression, the deleterious effects of inbreeding, may not be initially seen in a population and a 'wait and see' approach to management may have disastrous effects (Frankham 1996, Frankham 2005).
4. Maintenance of historical processes, as identified by genetic and ecological analysis (Moritz 1999; Crandall *et al.* 2000; Moritz 2002). These processes can be maintained by using the evolutionary significant unit (ESU) and management unit (MU) concept (Moritz 1999; Moritz 2002). Results from our microsatellite analyses indicate that each identified sampling group can be considered an MU (Moritz 1999; Crandall *et al*. 2000; Palsbøll *et al*. 2007), however translocation history of populations in South Africa (see Garaï *et al*. 2004; Chapter 1) support the combination of the following groups into one MU: KNP, AENP, Pilanesberg group (including PNP, Phinda and Zimbabwe), HiP and Madikwe. Despite AENP's significant genetic divergence from other populations in South Africa, this has been due to genetic drift as a result of anthropogenic influences (Whitehouse & Harley 2001) and historical "genetic exchangeability" should be restored (Crandall *et al*. 2000) as proposed by Whitehouse and Harley (2001). Our data further support Botswana and Zambia as a single MU and Namibia as a separate MU. The suggested MUs are based on microsatellite data and translocation history, and should be corroborated with the use of other genetic markers, for example mtDNA sequence data, and further ecological data (Taylor & Dizon 1999; Palsbøll *et al*. 2007). Van Aarde & Ferreira (2009) have recently suggested the use of "clusters of elephant conservation areas" as conservation units for future elephant conservation in southern Africa. These clusters were recognised using ecological data as well as the proximity of populations to each other. There is some similarity between these clusters and the MUs defined here; however these MU's tend to encompass more than one cluster (for example the MU encompassing South Africa and Zimbabwe is composed of the Limpopo and Maputo clusters in van Aarde & Ferreira (2009). This difference may suggest a lack of sufficient genetic data, related to sample sizes and/or genetic markers, or differences in ecological data which may not necessarily be reflected in genetic data.

In conclusion, the genetic diversity of reintroduced elephant populations at the microsatellite level is at healthy levels compared to one of the source populations. Despite past management decisions ignoring genetic principles, this does not mean they should be in the future. The AENP population is a clear example of how a population bottleneck can be detected genetically, and how a low number of founders resulted in genetic drift in the population over time (Whitehouse & Harley 2001). Further studies should be undertaken to investigate the genetic diversity of new generations of elephants in current parks to monitor diversity in these populations and identify and rectify any possible signs of genetic drift or inbreeding (Schwartz *et al*. 2007).

The addition of mtDNA control region sequences to previously published data sets provides signatures of historical movement between eastern and southern Africa. A broader investigation into mtDNA control region diversity of southern Africa will help to determine if there are any haplotypes which are unique to the region and further add to phylogeographic analyses of the African elephant.

Chapter 5

Conclusion

The debate on whether demographic or genetic factors will cause the extinction of a population (Lande 1988; Caughley 1994; Hedrick *et al*. 1996; DeSalle & Amato 2004; Frankham 2005) will continue for generations to come. Demographic factors follow an obvious line of thought for small, isolated populations; for example, if all the individuals born in a generation are of only one sex (Hedrick *et al*. 1996), no matter how hard we try to improve various factors, this population's extinction is guaranteed. With regards to genetic factors, there are now more and more examples of how small, isolated populations are suffering the effects of a loss in genetic diversity (reviewed in Spielmann *et al.* 2004; Frankham 2005; O'Grady *et al*. 2006). However a population's demography and its genes are not mutually exclusive, and may at times influence each other or even compound the effect of a single factor (Lacy 2000a). Therefore a combined approach interpreting these and other factors affecting a population's extinction risk needs to be included into future management decisions since ignoring any one factor will have potentially disastrous effects for the future survival of a population.

Small populations are known to suffer from a greater extinction risk due to demographic, genetic and stochastic factors (Shaffer 1981, Frankham 2005). One can expect reintroduced elephant populations to suffer from these problems, some of which have already been observed. For example: no sexually mature males present for a number of years resulting in a lack of population growth (see Whitehouse 2001), and a chance event resulting in the death of a large proportion of a population (see Woolley *et al*. 2008b). In this study, I looked at the demographic history of one of these reintroduced populations and investigated the genetic diversity of a number of populations (reintroduced and natural).

Despite our demographic analyses and that of Mackey *et al.* (2006) showing that most of these reintroduced populations are growing at rates much greater than that of more natural populations, it does not mean that these populations are immune to stochastic factors. The sensitivity of these small populations to catastrophic stochastic events was highlighted by the fire that swept through the Pilanesberg National Park in 2005 and killed 29 individuals (18% of the populations, Woolley *et al*. 2008b).

Future projections of the Pilanesberg population indicated growth rates lower than historical rates, but still higher than Calef's (1988) theoretical maximum growth rate. However, the age structure appeared

to start stabilizing and the sex ratio approached a 1:1 ratio. Intercalving interval was the most sensitive demographic factor, and future research should investigate management regimes, for example contraception (Delsink *et al*. 2006), which could achieve this. Harvesting of adult males was found to be an effective short-term management option for managing population growth, however the effectiveness of this method in reducing population size has recently been questioned (see Martin *et al*. 1996; Slotow *et al*. 2008). These results show how modelling can highlight which population growth factors need to be considered for future management plans.

The results of the genetic analyses in this study indicate that the microsatellite genetic diversity of many of these reintroduced populations showed similar levels of heterozygosity and greater allelic richness than that of the Kruger National Park population. The Addo Elephant National Park population, as in the Whitehouse and Harley (2001) study, was found to have suffered a loss in genetic diversity due its population bottleneck. This example clearly highlights the fact that genetic diversity can be lost through management. Management or even stochastic events (for example fires) can in future result in bottlenecks, founder effects, and genetic drift, eventually leading to inbreeding depression and a loss of evolutionary potential, ultimately increasing extinction risk (Lacy 1997; Meffe & Carrol 1997; Frankham 2005). The difference in diversity between the southern and eastern populations highlights the effects of demographic history on genetic diversity.

Due to their long-generation time it is unlikely that in our generation we will see the deleterious effects of management on a population's genetic diversity, however, Addo Elephant National Park is a prime example where these effects are visible. Fortunately there are now an increasing number of examples from species in the wild which provide support for genetic theory (Frankham 2005). This highlights the fact that genetic principles should not be ignored in management decisions. Therefore the basic principles of maintaining large population sizes, maintaining genetic diversity, allowing artificial gene flow, and the maintenance of historical processes (Frankham *et al*. 2002) can and should be applied in future elephant management plans and form part of an 'active adaptive management' plan (*sensu* Biggs *et al*. 2008).

Future Research

Population monitoring needs to continue at both the demographic and genetic scale. Regular analyses of data generated will provide early warning signs of factors affecting extinction risk. Analyses of demographic parameters should continue into the future, as well as population modelling of the Pilanesberg National Park and other reintroduced populations (Mackey *et al*. 2006). With these predictions management plans should be developed to deal with these growing populations and in turn

be analysed within simulations before being placed into action (Owen-Smith *et al*. 2006). Non-invasive DNA sampling from dung should be re-evaluated with more modern techniques and greater experience that are now available with regards to handling these samples (Broquet *et al*. 2007; Eggert *et al*. 2008; Beja-Pereira *et al*. 2009). Genetic monitoring (Schwartz *et al*. 2007) of successive generations will allow us to determine if there is any loss of genetic diversity and allow for corrective management. Despite the development of a myriad of microsatellite markers (Nyakaana & Arctander 1998, Comstock *et al*. 2000, Eggert *et al*. 2000, Archie *et al*. 2003, Nyakaana *et al*. 2005), research groups should use the same loci in order to improve comparisons between studies. MtDNA control region sequences should be determined for a larger sample of Southern African elephants, and analysed with previously published data to determine the presence of two distinct haplogroups among African 'savanna' elephants. Finally any data obtained from these reintroduced populations, ecological, demographic, genetic, behavioural or other, should be used in combination to develop 'active adaptive management' plans (*sensu* Biggs *et al*. 2008) for the multiple threats that increase these population's extinction risks.

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Appendix 1 Population data for the Pilanesberg elephant population for the period 1979-2003. Includes number of individuals introduced, number of individuals that died or were removed, number of known births as well as the number of individuals estimated from the total population counts. Blanks indicate data is unknown. Juv stands for Juveniles (2-6 years old), Sub-Ad for Sub Adult (6-15 years), yrs for years.

Year	Introductions				Removals / Deaths				Births	Population size
	Females	Age	Males	Age	Females	Age	Males	Age		
1979	2	juv	3	juv	2	Juv	2	Juv		1
1980										1
1981	5	juv	13	juv						19
1982	2	19yrs					8	Juv		13
1983	12	juv	14	juv			1	Juv		39
1984							1	Juv		38
1985										38
1986										38
1987							2	Juv		36
1988										36
1989									1	37
1990									1	38
1991							1	Sub-Ad	1	38
1992			2	18yrs						
1993	17	juv	19	juv			4	Sub-Ad		
1994							3	Adult		
1995										
1996							3	Adult		
1997										
1998			6	20yrs						96
1999										
2000							3	Adult		
2001							2	Adult	6	141
2002							2	Adult		
2003										162

Appendix 2a 1998 Population structure, used for simulations in both the Simulele programme and VORTEX. In VORTEX females were modelled per age class, the model does not include biological states.

Age	Males	Immature Females	Females- not pregnant	Females- 1st year pregnancy	Females- 2nd year pregnancy	Females- 1st year of anoestrous
1	3	4	0	0	0	0
2	3	3	0	0	0	0
3	1	1	0	0	0	0
4	0	1	0	0	0	0
5	1	1	0	0	0	0
6	4	4	0	0	0	0
7	0	1	0	0	0	0
8	0	1	0	0	0	0
9	1	0	0	0	0	0
10	1	1	1	0	0	0
11	3	2	1	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	2	0	2	0	0	1
16	1	0	0	1	1	0
17	1	0	0	1	2	0
18	1	0	1	2	1	0
19	2	0	0	2	2	0
20	2	0	0	1	2	1
21	2	0	0	2	1	0
22	1	0	0	1	2	0
23	3	0	0	2	1	1
24	0	0	0	1	1	0
25	2	0	0	0	0	1
26	0	0	0	0	0	0
27	0	0	0	0	0	0
28	0	0	0	0	0	0
29	0	0	0	0	0	0
30	6	0	0	0	0	0
31	0	0	0	0	0	0
32	0	0	0	0	0	0
33	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	2	0	0	0

Appendix 2b 1989 Population structure, used for simulations in both the Simulele programme and VORTEX. In VORTEX females were modelled per age class, the model does not include biological states.

Age	Males	Immature Females	Females- not pregnant	Females- 1st year pregnancy	Females- 2nd year pregnancy	Females- 1st year of anoestrous
1	1	0	0	0	0	0
2	2	2	0	0	0	0
3	3	3	0	0	0	0
4	8	7	0	0	0	0
5	4	3	0	0	0	0
6	2	2	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	6	2	3	0	0	0
11	5	1	3	0	0	0
12	2	0	2	0	0	0
13	1	0	1	0	0	0
14	2	0	1	0	0	0
15	1	0	1	1	1	1
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0
21	0	0	0	0	0	0
22	0	0	0	0	0	0
23	0	0	0	0	0	0
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	0	0	2	0	0	0

Appendix 2c 2003 Population structure, used for simulations in both the Simulele programme and VORTEX. In VORTEX females were modelled per age class, the model does not include biological states.

Age	Males	Immature Females	Females- not pregnant	Females- 1st year pregnancy	Females- 2nd year pregnancy	Females- 1st year of anoestrous
1	6	6	0	0	0	0
2	3	3	0	0	0	0
3	3	2	0	0	0	0
4	3	3	0	0	0	0
5	4	4	0	0	0	0
6	4	4	0	0	0	0
7	1	2	0	0	0	0
8	2	1	0	0	0	0
9	2	1	1	0	0	0
10	2	1	1	0	0	0
11	1	1	1	0	0	0
12	2	1	1	1	0	0
13	2	0	2	1	1	0
14	2	0	1	1	1	0
15	0	0	2	2	2	2
16	4	0	2	1	1	1
17	7	0	1	2	1	1
18	5	0	1	1	2	1
19	4	0	1	1	1	2
20	0	0	1	0	1	0
21	0	0	0	1	0	0
22	0	0	0	0	1	1
23	0	0	1	0	0	0
24	1	0	1	1	1	0
25	1	0	0	1	1	1
26	0	0	0	0	1	1
27	2	0	1	0	1	0
28	1	0	0	1	1	1
29	1	0	1	1	0	0
30	1	0	1	0	0	0
31	0	0	0	0	0	0
32	0	0	0	0	0	0
33	2	0	0	0	0	0
34	0	0	0	0	0	0
35	1	0	0	0	0	0
36	1	0	0	0	0	0
37	0	0	0	0	0	0
38	0	0	0	0	0	0
39	0	0	0	0	0	0
40	1	0	0	0	0	2

Appendix 3a Sensitivity analyses for varying sex ratios at birth, for the period 1998 to 2003. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1998 to 2003 ($r = 0.1065$) are in bold (based on t-tests comparing two slopes (Zar 1996).

Mean intercalving interval	Sex ratio	Simulated rates of increase 1998 - 2003[#]		
	females:males	*Leslie	*Wu & Botkin	*VORTEX
3 years	50:50	0.103	0.105	0.100
	60:40	0.103	0.105	0.103
	70:30	0.103	0.105	0.102
	40:60	0.103	0.105	0.101
	30:70	0.103	0.105	0.103
4 years	50:50	**0.081**	0.084	0.082
	60:40	**0.081**	0.084	**0.080**
	70:30	**0.081**	0.084	0.082
	40:60	**0.081**	0.084	0.082
	30:70	**0.081**	0.085	**0.081**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) vs time.

*__Leslie__ refers to the Leslie matrix model calculated using the Simulele modelling programme. **Wu & Botkin** refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. **VORTEX** refers to Lacy *et al.*'s (2003) population modelling programme.

Appendix 3b Sensitivity analyses of death rates for the adult age group, for the period 1998 to 2003. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1998 to 2003 ($r = 0.1065$) are given in bold (based on t-tests comparing two slopes (Zar 1996).

Mean intercalving interval	% Death rate	Simulated rate of increase 1998 - 2003[#]		
		*Leslie	*Wu & Botkin	*VORTEX
3 years	0	0.103	0.105	0.100
	0.5	0.100	0.103	0.101
	1	0.097	0.100	0.101
	1.5	0.094	0.096	0.102
	5	**0.075**	**0.077**	0.095
4 years	0	**0.081**	0.084	0.082
	0.5	**0.079**	0.081	**0.082**
	1	**0.076**	0.078	**0.079**
	1.5	**0.073**	0.077	**0.079**
	5	**0.054**	**0.058**	**0.074**

[0#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*__Leslie__ refers to the Leslie matrix model calculated using the Simulele modelling programme. **Wu & Botkin** refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. **VORTEX** refers to Lacy *et al.*'s (2003) population modelling programme.

Appendix 4a Sensitivity analyses for various adult mortality rates, for the period 1989 to 1998. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1989 to 1998 (r = 0.0381) are in bold (based on t-tests comparing two slopes (Zar 1996).

Mean intercalving interval	Mean age at first calving	Simulated rates of increase 1989 - 1998[#]				
		% Death rate				
		0	0.5	1	2.5	5
				*Leslie		
	10	**0.052**	**0.051**	**0.050**	**0.046**	0.041
5 years	12	0.046	0.044	0.043	0.039	0.033
	14	0.039	0.039	0.038	0.034	**0.028**
	10	0.045	0.044	0.043	0.039	0.033
6 years	12	0.039	0.038	0.037	0.033	**0.027**
	14	0.035	**0.033**	0.032	**0.028**	**0.022**
	10	0.039	0.039	0.037	0.034	**0.028**
7 years	12	0.035	0.033	0.032	**0.028**	**0.022**
	14	**0.030**	**0.029**	**0.027**	**0.024**	**0.017**
				*Wu & Botkin		
	10	**0.054**	**0.053**	**0.052**	**0.048**	0.042
5 years	12	**0.049**	**0.048**	0.047	0.043	0.038
	14	0.046	0.045	0.044	0.041	0.033
	10	0.046	0.044	0.042	0.039	0.033
6 years	12	0.041	0.039	0.039	0.034	**0.028**
	14	0.039	0.037	0.036	0.033	**0.027**
	10	0.039	0.038	0.037	0.033	**0.026**
7 years	12	0.036	0.034	0.032	**0.029**	**0.023**
	14	0.034	0.032	**0.031**	**0.027**	**0.022**
				*VORTEX		
	10	**0.051**	**0.050**	**0.047**	0.040	**0.030**
5 years	12	0.044	0.041	0.040	**0.033**	**0.022**
	14	0.036	0.034	0.032	**0.028**	**0.019**
	10	0.044	0.040	0.039	0.033	**0.022**
6 years	12	0.035	0.034	**0.032**	**0.026**	**0.015**
	14	**0.030**	**0.028**	**0.027**	**0.020**	**0.011**
	10	0.040	0.036	0.034	**0.027**	**0.017**
7 years	12	0.032	**0.029**	**0.029**	**0.021**	**0.011**
	14	**0.026**	**0.024**	**0.023**	**0.018**	**0.008**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*__Leslie__ refers to the Leslie matrix model calculated using the Simulele modelling programme. __Wu & Botkin__ refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. __VORTEX__ refers to Lacy *et al.*'s (2003) population modelling programme.

Appendix 4b Sensitivity analyses for various sub-adult (6 - 15 years old) mortality rates, for the period 1989 to 1998. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1989 to 1998 ($r = 0.0381$) are in bold (based on t-tests comparing two slopes (Zar 1996).

Mean intercalving interval	Mean age at first calving	Simulated rates of increase 1989 - 1998[#]				
		% Death rate				
		0	0.5	1	2.5	5
				*Leslie		
	10	**0.052**	**0.051**	**0.050**	0.046	0.039
5 years	12	0.046	0.044	0.043	0.039	0.032
	14	0.039	0.039	0.037	0.033	**0.026**
	10	0.045	0.044	0.042	0.038	0.031
6 years	12	0.039	0.038	0.036	0.032	**0.025**
	14	0.035	0.033	0.031	**0.027**	**0.020**
	10	0.039	0.038	0.037	0.033	**0.026**
7 years	12	0.035	0.033	0.031	**0.027**	**0.020**
	14	**0.030**	**0.029**	**0.027**	**0.023**	**0.016**
				*Wu & Botkin		
	10	**0.054**	**0.053**	**0.051**	0.047	0.040
5 years	12	**0.049**	**0.049**	0.046	0.042	0.035
	14	0.046	0.045	0.044	0.039	0.032
	10	0.046	0.044	0.042	0.038	0.032
6 years	12	0.041	0.040	0.038	0.033	**0.028**
	14	0.039	0.038	0.037	0.032	**0.025**
	10	0.039	0.037	0.037	0.032	**0.024**
7 years	12	0.036	0.034	0.032	**0.029**	**0.022**
	14	0.034	0.032	0.031	**0.026**	**0.019**
				*VORTEX		
	10	**0.051**	**0.050**	**0.048**	0.042	0.032
5 years	12	0.044	0.043	0.043	0.041	0.038
	14	0.036	0.036	0.033	0.032	**0.029**
	10	0.044	0.041	0.039	0.033	**0.021**
6 years	12	0.035	0.035	0.036	0.034	**0.031**
	14	**0.030**	**0.029**	**0.028**	**0.026**	**0.021**
	10	0.040	0.037	0.035	**0.028**	**0.019**
7 years	12	0.032	0.032	**0.031**	**0.030**	**0.027**
	14	**0.026**	**0.024**	**0.024**	**0.022**	**0.018**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*__Leslie__ refers to the Leslie matrix model calculated using the Simulele modelling programme. __Wu & Botkin__ refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. __VORTEX__ refers to Lacy *et al.*'s (2003) population modelling programme.

Appendix 4c Sensitivity analyses for combined adult and sub-adult mortality rates, for the period 1989 to 1998. Annual rates of increase (r) as simulated by the population models are shown. Rates that are significantly different ($p < 0.05$) from the actual observed rate of increase for 1989 to 1998 (r = 0.0381) are in bold (based on t-tests comparing two slopes (Zar 1996).

Mean intercalving interval	Mean age at first calving	Simulated rates of increase 1989 - 1998[#]				
		% Adult and Sub-adult death rate				
		0	0.5	1	2.5	5
				*Leslie		
	10	**0.052**	**0.050**	**0.047**	0.040	**0.027**
5 years	12	0.046	0.043	0.040	0.032	**0.020**
	14	0.039	0.037	0.035	**0.027**	**0.014**
	10	0.045	0.043	0.040	0.032	**0.020**
6 years	12	0.039	0.037	0.034	**0.026**	**0.013**
	14	0.035	0.032	**0.029**	**0.021**	**0.008**
	10	0.039	0.037	0.034	**0.027**	**0.014**
7 years	12	0.035	0.032	**0.029**	**0.021**	**0.008**
	14	**0.030**	**0.027**	**0.025**	**0.016**	**0.004**
				*Wu & Botkin		
	10	**0.054**	**0.051**	**0.049**	0.040	0.029
5 years	12	**0.049**	0.046	0.044	0.036	**0.024**
	14	0.046	0.044	0.041	0.034	**0.019**
	10	0.046	0.043	0.040	0.033	**0.019**
6 years	12	0.041	0.039	0.036	**0.026**	**0.015**
	14	0.039	0.037	0.034	**0.026**	**0.011**
	10	0.039	0.036	0.034	**0.025**	**0.013**
7 years	12	0.036	0.033	0.031	**0.022**	**0.009**
	14	0.034	0.031	**0.028**	**0.020**	**0.006**
				*VORTEX		
	10	**0.051**	**0.049**	**0.045**	0.039	**0.027**
5 years	12	0.044	0.041	0.038	0.032	**0.018**
	14	0.036	0.034	**0.030**	**0.023**	**0.010**
	10	0.044	0.041	0.037	**0.031**	**0.018**
6 years	12	0.035	0.033	**0.031**	**0.024**	**0.010**
	14	**0.030**	**0.028**	**0.025**	**0.018**	**0.004**
	10	0.040	0.035	0.033	**0.027**	**0.013**
7 years	12	0.032	**0.031**	**0.027**	**0.018**	**0.006**
	14	**0.026**	**0.025**	**0.020**	**0.013**	**0.001**

[#] Rates of increase were calculated from the slope of the regression line of ln (population estimate) versus time.

*__Leslie__ refers to the Leslie matrix model calculated using the Simulele modelling programme. **Wu & Botkin** refers to the modified Wu and Botkin (1980) model as calculated by the Simulele modelling programme. **VORTEX** refers to Lacy *et al.*'s (2003) population modelling programme.

Appendix 5 Reproductive parameters of female elephants in various populations throughout Africa.

Location	Mean age at first calving (years)	Mean intercalving interval (years)	Source
Kruger National Park	12 to 13	4.0	Whyte (2001)
Addo Elephant National Park	12.3	3.3	Gough & Kerley (2006)
Gonarezhou National Park, Zimbabwe	14 to 15	4.0	Sherry (1975)
Hwange National Park, Zimbabwe	13	4.0	Williamson (1976)
Mana Pools National Park, Zimbabwe	14 to 15	3.5	Kerr (1978)
Luangwa Valley, Zambia	16	3.5 to 4.0	Hanks (1972)
Amboseli National Park, Kenya	13.67	4.5	Moss (2001)
Sweetwaters Game Reserve, Kenya	-	3.8	Ogola & Omondi (2005)
Tsavo East National Park, Kenya	14.5 (1969)	6.8 (1969)	Laws (1969)
	17 (2000)	5 (2000)	McKnight (2000)
Lake Manyara National Park, Tanzania	13	4.7	Douglas-Hamilton (1972)
Mkomasi Game Reserve, Tanzania	13	4.0 to 5.0	Laws (1969)
Murchinson Falls National Park North, Uganda	14	6.0 to 7.0	Laws (1969)
Kasungu National Park, Malawi	13	3.3	Jachmann (1986)
Liwonde National Park, Malawi	13	3	Bhima (1998)

Appendix 6 Permit to remove elephant dung from the National Zoological Gardens, Pretoria.

G.P.-S. LTD 2747

Permit in	Permit uit/out

Verw. Ref. V3/2001

REPUBLIEK VAN SUID-AFRIKA · REPUBLIC OF SOUTH AFRICA

DEPARTEMENT VAN LANDBOU-TEGNIESE DIENSTE
DEPARTEMENT OF AGRICULTURAL TECHNICAL SERVICES

PERMIT VIR VERVOER VAN DIERE/DIERLIKE OF PLANTAARDIGE PRODUKTE
PERMIT TO MOVE ANIMALS/ANIMAL OR PLANT PRODUCTS

Kragtens die Wet op Dieresiektes en -parasiete, 1956 (Wet 13 van 1956), soos gewysig, en onderworpe aan die voorwaardes hieronder gestel, word toestemming hiermee verleen aan—

In terms of the Animal Diseases and Parasites Act, 1956 (Act 13 of 1956), as amended and subject to the conditions specified below permission is hereby granted to—

Naam / Name: V3 Marina Alais.

Adres / Address: Univ of Pretoria.

Om te beweeg met/Vir die vervoer van / To move with/To transport: Elephant dung.

Van die plaas/plek / From the farm/place: Nat Zoo. in die distrik / in the district of: Pretoria.

Na die plaas/abattoir/plek / To the farm/abattoir/place: Univ of Pretoria. in die distrik / in the district of: Pretoria.

Voorwaardes

1. Hierdie permit—
   (a) is geldig vir 30 dae vanaf datum van uitreiking en slegs vir een beweging;
   (b) moet die diere/produkte hierbo vermeld vergesel en moet vir inspeksie getoon word op die versoek van enige eienaar van grond of vee, 'n lid van die Suid-Afrikaanse Polisie of 'n beampte van die Departement van Landbou-Tegniese Dienste;
   (c) dien nie as magtiging om te beweeg met diere wat in kwarantyn verkeer of diere wat besmet of vermoedelik besmet is met 'n siekte of uitwendige parasiete nie;
   (d) moet by die plek van bestemming gehou word totdat dit deur 'n gemagtigde persoon opgeëis word.
2. Diere afkomstig van gespesifiseerde beperkte gebiede moet in verseëlde spoorwegtrokke/vervoermiddels vervoer word.
3. Tydens vervoer moet alle kwarantyngebiede wat besmet of vermoedelik besmet is met 'n siekte wat sodanige diere aantas, vermy word.
4. Huide en velle van gespleethoewige diere afkomstig van gespesifiseerde beperkte gebiede, moet onder amptelike toesig in verseëlde spoorwegtrokke gelaai word en die seels mag oorbestemming alleen deur 'n gemagtigde beampte gebreek word.
5. Room afkomstig van gespesifiseerde beperkte gebiede mag alleenlik na vooraf goedgekeurde romerye in verseëlde roomkanne gestuur word.
6. Ander voorwaardes.....

Conditions

1. This permit—
   (a) is valid for 30 days from date of issue and for one movement only;
   (b) must accompany the animals/products mentioned above and must be produced for inspection on demand by any land owner, stock owner, member of the South African Police or any officer of the Department of Agricultural Technical Services;
   (c) does not authorise the removal of animals in quarantine or animals infected or suspected of being infected with a disease or infested or suspected of being infested with external parasites;
   (d) must be kept at the place of destination until it is collected by an authorised person.
2. Animals originating from specified restricted areas must be conveyed in sealed railway trucks/mechanical transport.
3. The route followed must avoid all quarantined areas and areas infected or suspected of being infected with any disease to which the animals are susceptible.
4. Hides and skins of cloven hoofed animals originating in specified restricted areas must be loaded under official supervision into sealed railway trucks, and the seals may only be broken at destination by an authorised officer.
5. Cream originating in specified restricted areas may only be moved to previously approved creameries in sealed cans.
6. Other conditions.....

DEPT. LANDBOU, BEWARING EN OMGEWING
STAATSVEEARTS
PRIVAATSAK/PRIVATE BAG X 369
PRETORIA 0001
STATE VETERINARIAN
DEPT. OF AGRICULTURE, CONSERVATION & ENVIRONMENT

Plek / Place: Pretoria. Datum / Date: 07/03/2001

Staatsveearts/State Veterinarian

VERSPREIDING/DISTRIBUTION

Die / The .....

Die / The .....

Die / The .....

Hiermee word gesertifiseer dat ek:
I hereby certify that:

(a) ..... beeste/skape/bokke/varke geïnspekteer/gebek en cattle/sheep/goats/pigs were inspected/mouthed by me and found free of.....;

(b) die produkte hierbo vermeld ondersoek het, en tevrede is dat geen risiko van verspreiding van siekte bestaan nie.
I have examined the products mentioned above, and satisfied myself that no risk of dissemination of disease exists.

Staatsveearts/State Veterinarian
Veeïnspekteur/Stock inspector

Appendix 7 Reference list of articles published in *Molecular Ecology* (1992-2009) and *Conservation Genetics* (2000-2009) using faecal DNA, excludes reviews.

Adams JR, Kelly BT, Waits LP (2003) Using faecal DNA sampling and GIS to monitor hybridization between red wolves (*Canis rufus*) and coyotes (*Canis latrans*). *Molecular Ecology*, **12**, 2175-2186.

Adams JR, Lucash C, Schutte L, Waits LP (2007) Locating hybrid individuals in the red wolf (*Canis rufus*) experimental population area using a spatially targeted sampling strategy and faecal DNA genotyping. *Molecular Ecology*, **16(9)**, 1823-1834.

Adams JR, Waits LP (2007) An efficient method for screening faecal DNA genotypes and detecting new individuals and hybrids in the red wolf (*Canis rufus*) experimental population area. *Conservation Genetics*, **8(1)**, 123-131.

Ball MC, Pither R, Manseau M, Clark J, Petersen SD, Kingston S, Morrill N, Wilson P (2007) Characterization of target nuclear DNA from faeces reduces technical issues associated with the assumptions of low-quality and quantity template. *Conservation Genetics*, **8(3)**, 577-586.

Banks SC, Horsup A, Wilton AN, Taylor AC (2003) Genetic marker investigation of the source and impact of predation on a highly endangered species. *Molecular Ecology*, **12**, 1663-1667.

Bayes MK, Smith KL, Alberts SC, Altmann J, Bruford MW (2000) Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. *Conservation Genetics*, **1**, 173-176.

Bidlack AL, Reed SE, Palsbøll PJ, Getz WM (2007) Characterization of a western North American carnivore community using PCR–RFLP of cytochrome *b* obtained from fecal samples. *Conservation Genetics*, **8(6)**, 1511-1513.

Bowkett AE, Plowman AM, Stevens JR, Davenport TRB, Jansen van Vuuren B (2009) Genetic testing of dung identification for antelope surveys in the Udzungwa Mountains, Tanzania. *Conservation Genetics*, **10(1)**, 251-255.

Bradley BJ, Boesch C, Vigilant L (2000) Identification and redesign of human microsatellite markers for genotyping wild chimpanzee (*Pan troglodytes verus*) and gorilla (*Gorilla gorilla gorilla*) DNA from faeces. *Conservation Genetics*, **1**, 289-292.

Bradley BJ, Chambers KE, Vigilant L (2001) Accurate DNA-based sex identification of apes using non-invasive samples. *Conservation Genetics*, **2**, 179-181.

Brown SM, Houlden BA (2000) Conservation genetics of the black rhinoceros (*Diceros bicornis*). *Conservation Genetics*, **1**, 365-370.

Chu J-H, Lin Y-S, Wu H-Y, (2005) Mitochondrial DNA diversity in two populations of Taiwanese macaque (*Macaca cyclopis*).*Conservation Genetics*, **6(1)**, 101-109.

Constable JL, Ashley MV, Goodall J, Pusey AE (2001) Noninvasive paternity assignment in Gombe chimpanzees. *Molecular Ecology*, **10**, 1279-1300.

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

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Appendix 8 MtDNA control region sequences obtained from GenBank used, in combination with sequences obtained in this study, to further investigate relationships between populations in southern and eastern Africa. n/a – not available.

GenBank reference number	Haplotype name	Country of origin	Nature reserve	Reference
AF 106222	ZBE1	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106223	ZBE2	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106224	ZBE3	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106225	ZBE4	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106226	ZBE5	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106227	ZBE6	Zimbabwe	n/a	Nyakaana *et al*. 2002
AF 106228	BOT2	Botswana	Chobe National Park	Nyakaana *et al*. 2002
AF 106229	BOT4	Botswana	Chobe National Park	Nyakaana *et al*. 2002
AF 106230	BOT6	Botswana	Chobe National Park	Nyakaana *et al*. 2002
AF 106231	BOT9	Botswana	Kwando	Nyakaana *et al*. 2002
AF 106232	BOT15	Botswana	Ngwasha	Nyakaana *et al*. 2002
AF 106233	BOT16	Botswana	Nunga Valley	Nyakaana *et al*. 2002
AF 106234	BOT21	Botswana	Sibuyu Forest Reserve	Nyakaana *et al*. 2002
AF 106235	WC2	Namibia	Western Caprivi Recreation Park	Nyakaana *et al*. 2002
AF 106236	WC4	Namibia	Western Caprivi Recreation Park	Nyakaana *et al*. 2002
AF 106237	WC6	Namibia	Western Caprivi Recreation Park	Nyakaana *et al*. 2002
AF 106238	WC13	Namibia	Western Caprivi Recreation Park	Nyakaana *et al*. 2002
AF 106239	KH2	Namibia	Khorixis	Nyakaana *et al*. 2002
AF 106240	KG1	South Africa	Kruger National Park	Nyakaana *et al*. 2002
AF 106241	KG2	South Africa	Kruger National Park	Nyakaana *et al*. 2002
AF 527681	Addo1	South Africa	Addo Elephant National Park	Eggert *et al*. 2002
AF 527682	Addo5	South Africa	Addo Elephant National Park	Eggert *et al*. 2002
AF 106203	KV1	Uganda	Kidepo Valley National Park	Nyakaana *et al*. 2002
AF 106204	KV2	Uganda	Kidepo Valley National Park	Nyakaana & Arctander 1999
AF 106205	KV7	Uganda	Kidepo Valley National Park	Nyakaana & Arctander 1999
AF 106206	KV8	Uganda	Kidepo Valley National Park	Nyakaana & Arctander 1999

Appendix 8 continued

GenBank reference number	Haplotype name	Country of origin	Nature reserve	Reference
AF 106207	KV17	Uganda	Kidepo Valley National Park	Nyakaana & Arctander 1999
AF 106208	KV28	Uganda	Kidepo Valley National Park	Nyakaana & Arctander 1999
AF 106209	MF1	Uganda	Murchison Falls National Park	Nyakaana & Arctander 1999
AF 106210	MF5	Uganda	Murchison Falls National Park	Nyakaana & Arctander 1999
AF 106211	QE1	Uganda	Queen Elizabeth National Park	Nyakaana *et al*. 2002
AF 106212	QE4	Uganda	Queen Elizabeth National Park	Nyakaana & Arctander 1999
AF 106213	QE13	Uganda	Queen Elizabeth National Park	Nyakaana & Arctander 1999
AF 106214	MM4	Kenya	Masai Mara Game Reserve	Nyakaana *et al*. 2002
AF 106215	MM19	Kenya	Masai Mara Game Reserve	Nyakaana *et al*. 2002
AF 106216	MM20	Kenya	Masai Mara Game Reserve	Nyakaana *et al*. 2002
AF 106217	AM1	Kenya	Amboseli National Park	Nyakaana *et al*. 2002
AF 106218	AM2	Kenya	Amboseli National Park	Nyakaana *et al*. 2002
AF 106219	AM10	Kenya	Amboseli National Park	Nyakaana *et al*. 2002
AF 106220	AM12	Kenya	Amboseli National Park	Nyakaana *et al*. 2002
AF 106221	SA8	Kenya	Samburu National Park	Nyakaana *et al*. 2002
AF 527638	AF8	Kenya	n/a	Eggert *et al*. 2002
AF 527639	AF8	Kenya	n/a	Eggert *et al*. 2002
AF 527640	AF10	Kenya	n/a	Eggert *et al*. 2002
AF 219239	n/a	Tanzania	n/a	Unpublished, GenBank

Appendix 9 Allelic frequencies per sampling region for all microsatellite loci. Sample names: LKT - Kruger National Park, A - Addo Elephant National Park, LPT - Pilanesberg National Park, LHB - Hluhluwe-iMfolozi Park, LMT - Madikwe Game Reserve, LNT - Phinda Game Reserve, LEH - Namibia, LZH - Zambia, LB - Botswana, LWH - Zimbabwe.

Locus	**Sampling Regions**						
	LKT	A	LPT	LHB	LMT	LEH	LZH
FH19							
185	0.07	0.02	0.15	0.13	0.29		
187	0.59	0.19	0.49	0.33	0.41	0.58	0.36
189	0.02	0.26	0.04	0.10		0.08	0.04
191			0.02	0.03	0.02		
193			0.04	0.10	0.02	0.17	0.18
195	0.19	0.52	0.18	0.23	0.17	0.17	0.39
197	0.07		0.06	0.10	0.09		
199	0.06		0.01				0.04
FH39							
234	0.15		0.02	0.15	0.02	0.17	
236					0.03		
238			0.01		0.09		0.04
240				0.10			
242	0.57		0.42	0.40	0.34	0.17	0.46
244	0.19	0.12	0.10	0.05	0.05	0.08	0.18
246	0.07	0.14	0.27	0.18	0.24	0.25	0.21
250			0.02		0.03	0.17	
252	0.02		0.03	0.05	0.02	0.08	0.04
254			0.01		0.02		
256		0.74	0.07	0.08	0.16	0.08	0.07
258			0.04				

Appendix 9 continued

Locus	Sampling Regions						
	LKT	A	LPT	LHB	LMT	LEH	LZH
LA5							
141				0.08	0.02	0.10	
143	0.21		0.40	0.03	0.17		0.25
145			0.02	0.65		0.20	
147	0.69	1.00	0.45	0.20	0.78	0.40	0.68
149	0.04		0.01	0.03			
151			0.01			0.20	0.07
153	0.02		0.01	0.03			
155	0.04		0.10		0.03	0.10	
LA6							
160				0.03		0.33	
164				0.03	0.12		
166	0.21	1.00	0.28	0.23	0.26	0.42	0.53
172	0.04		0.03	0.18	0.21		
174	0.75		0.68	0.55	0.36	0.25	0.47
176			0.01				
178					0.05		
LafMS02							
143	0.03		0.02		0.05		
145	0.03	0.10	0.14	0.05	0.10		
147	0.29	0.43	0.20	0.18	0.29	0.50	0.40
149	0.35	0.33	0.20	0.40	0.28	0.50	0.40
151	0.18	0.14	0.11	0.25	0.07		0.20
155	0.12		0.34	0.13	0.21		

Appendix 9 continued

Locus	Sampling Regions						
	LKT	A	LPT	LHB	LMT	LEH	LZH
LafMS03							
142	0.06	0.33	0.09	0.05		0.50	
144	0.56	0.67	0.64	0.68	0.69		0.61
146	0.38		0.224	0.28	0.31		0.36
148			0.02				0.04
150						0.50	
LafMS04							
154	0.04		0.01				
156	0.11		0.11	0.10	0.21	0.25	0.07
158	0.83	0.93	0.83	0.88	0.79	0.67	0.87
160	0.02	0.07	0.02	0.03		0.08	0.07
LafMS05							
154	0.93	1.00	0.98	0.93	0.97	1.00	1.00
156	0.04						
160			0.02	0.08	0.03		
162	0.04						
FH67							
94			0.01		0.03	0.25	
96	0.05		0.07		0.03		0.06
98	0.55	0.95	0.49	0.38	0.50	0.42	0.50
100	0.11		0.14	0.34	0.21	0.33	0.17
102	0.11		0.08		0.06		0.11
104			0.06	0.03			0.11
106	0.16		0.08	0.16	0.09		0.06
108	0.03		0.04	0.03	0.09		
110			0.01	0.06			
112			0.01				

Appendix 10

Published mean expected heterozygosity (Exp Het), observed heterozygosity (Obs het), mean number of alleles per locus (alleles/locus) for southern ([#]) and eastern ([Δ]) African and Asian (*) elephant populations. n/a – data not available.

Population/ Country	Sample size	Exp Het	Obs Het	Alleles/locus
Addo Elephant National Park[2#]	105	0.18	0.19	1.89
Kruger National Park[2#]	108	0.44	0.42	3.89
Kruger National Park[3#]	12	0.51	0.59	3.75
Kruger National Park[4#]	11	n/a	0.63	4.6
Knysna, South Africa[6#]	5	n/a	0.56	3.5
Namibia[3#]	21	0.60	0.65	4.75
Namibia[4#]	8	n/a	0.69	5.1
Botswana[3#]	22	0.61	0.73	5
Botswana[4#]	33	n/a	0.62	4.4
Zimbabwe[3#]	6	0.59	0.71	3
Zimbabwe[4#]	20	n/a	0.62	4.6
Uganda[1 Δ]	41	0.70	0.77	5.9
Uganda[3 Δ]	86	0.70	0.78	6.1
Kenya[3 Δ]	40	0.67	0.77	4.6
Kenya[4 Δ]	31	n/a	0.64	4.3
Kenya[9 Δ]	100	0.75	0.69	6.1
Tanzania[4 Δ]	29	n/a	0.62	4.6
Tanzania[7 Δ]	76	0.71	0.70	7.5
India[5*]	295	0.51	0.45	2.7
Vietnam[8*]	17	0.44	0.36	2.3

Sources: [1] = Nyakaana & Arctander 1999, [2] = Whitehouse & Harley 2001, [3] = Nyakaana *et al.* 2002, [4] = Comstock *et al.* 2002, [5] = Vidya *et al.* 2005b, [6] = Eggert *et al.* 2008, [7] = Ishengoma *et al.* 2007, [8] = Vidya *et al.* 2007, [9] = Okello *et al.* 2008a.

Appendix 11

Published nucleotide and haplotype diversities for the mitochondrial DNA control region in African and Asian elephants. n/a – data not available, * - Asian elephants.

Source	Overall nucleotide diversity (%)	Nucleotide diversity range (%)	Overall haplotype diversity	Haplotype diversity range
Nyakaana & Arctander 1999	1.4	0.5 – 2.4	n/a	n/a
Nyakaana *et al*. 2002	2.0	0.08 – 2.7	0.85	0.12 – 1.00
Johnson *et al*. 2007	3.4	n/a	0.98	n/a
Okello *et al*. 2008a	1.68	0.58 – 2.51	n/a	n/a
Fernando *et al*. 2000*	1.7	0.2 – 1.6	0.87	0.49 – 0.86
Fleischer *et al*. 2001*	1.9	n/a	0.97	n/a
Vidya *et al*. 2005b*	1.2	0.1 – 0.6	0.67	0.08 – 0.67
Vidya *et al*. 2007*	0.2	n/a	0.70	n/a