DETERMINATION OF GENETIC DIVERSITY AND PATERNITY ANALYSIS OF ELEPHANTS IN TARANGIRE NATIONAL PARK USING GENOMIC DNA MICROSATELLITES

BY

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ABSTRACT

A study was conducted on elephants of Tarangire National Park (TNP) in Tanzania with the aim of determining their genetic diversity and paternity using elephant specific genomic DNA microsatellite markers. DNA was extracted from faecal samples collected from known elephants in the northern (n=61) and 12 adult males as well as unidentified females from Central (n=8) and Southern (n=6) subpopulations. The faecal samples were preserved either in absolute ethanol or silica prior to DNA extraction. The extracted DNA was PCR-amplified in duplicates using eight highly polymorphic microsatellite markers and PCR products were subsequently analysed on ABI 3100 Genetic Sequencer Analyser and alleles were scored using ABI Prism® GeneScan® analysis software. The obtained data was analysed using CERVUS program. The amplification and genotyping success per locus for both ethanol and silica-preserved samples ranged from 81.40 to 91.90% (n=86). All 8 microsatellite markers were polymorphic with a total of 60 alleles in the sampled population and the mean number of alleles per locus being 7.50. The number of alleles per locus ranged from 3 to 11 while the mean polymorphic information content (PIC) was 66%. The observed heterozygosity varied from 51.90 to 80.90% and the expected heterozygosity per locus ranged from 53.80 to 82.0% while the mean expected heterozygosity for all loci was 70.60%. Test for Hardy-Weinberg equilibrium (HWE) was not significant for all loci. The genotype data enabled assignment of 38% and 83% of offspring (n=29) at 80% confidence level to their fathers using 30% (12/40) and 100% (12/12) simulation, respectively with respect to the proportion of the sampled breeding males within the TNP. Further assessment of mating success among bulls in the Park revealed that 7.50% of all potential breeding bulls were

responsible for fathering 31.0% and 52% of all 29 offspring at 30% and 100% simulation, respectively. This study provides for the first time an important genetic database that has wide applications in future studies of African elephants in TNP and other conservation areas in Tanzania. The data obtained will be used to assess the genetic relatedness of elephants residing in the park as well assist in providing an insight on the effects of poaching on the current elephant social structure in TNP. This information will imperatively be useful in the understanding and conserving the endangered African elephants in different range states in Africa.

DECLARATION

I, Deusdedith Rwegasira Simon Ishengoma, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has not been submitted for a degree award at any other university.

Signature: .....

Date: 7/8/2003

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ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µL	Microlitre
µM	Micromoles
A	Adenine
ABI	Applied Biosystems
Bp	Base pair (s)
C	Cytosine
CITES	Convention on International trade of Endangered Species
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
EDT	Ethylene Diamine Tetra-acetic Acid
G	Guanine
gm	Gram
GR	Game Reserve
h	hour
Kb	Kilo base (s)
M	Molar
min	minute
mM	Millimole
NaI/TBE	Sodium Iodide/Tris-Boric acid/EDTA
NCAA	Ngorongoro Conservation Area Authority
ng	Nanogrammes

NP	National Park
^0C	degree Celsius
PA	Protected Area
PCR	Polymerase Chain Reaction
SUA	Sokoine University of Agriculture
S	Second
T	Thymine
Ta	Annealing temperature
TANAPA	Tanzania National Parks
Taq	*Thermus aquaticus*
TAWIRI	Tanzania Wildlife Research Institute
TBE	Tris Boric Acid and EDTA
TNP	Tarangire National Park
U	Units
UK	United Kingdom
USA	United States of America
V	Volt
v/v	Volume by volume
w/v	Weight by volume

CHAPTER ONE

1.0 INTRODUCTION

Wildlife conservation in Africa started during colonial times when laws were enacted to protect royal hunting grounds with particular species of animals for the rulers (Boshe, 1996). In Tanzania, wildlife conservation dates back to 1891 when German government enacted laws to control hunting. The laws were meant to regulate off-take, hunting methods, and trade in wildlife, with some endangered species being fully protected (MNRT, 1998).

After independence, African countries intensified conservation efforts by setting aside areas for wildlife conservation. For example, Tanzania increased it protected areas from 13 at the time of independence to the current 81 protected areas covering over 32% of its range land (Sabuni, G. personal communication, 2002). These areas are of different conservation status ranging from total conservation, where physical utilisation of wildlife is prohibited to partial conservation. In the latter category, sustainable utilisation of resources through systematic annual cropping of the resources is allowed except for endangered species such as elephants (MNRT, 1998).

The African elephant (*Loxodonta africana*) is the largest surviving land mammal currently found in 37 countries in the Sub-Saharan region (Barnes *et al.*, 1999). In these countries, elephants are only found in protected areas as opposed to the past when elephants ranged in all types of available habitat (Cumming *et al.*, 1990). The elephant population in Africa was estimated to be 1.3 million in 1979 but the number was reduced to almost half in a period between late 1979 up to late 1980s due to

poaching (Douglas-Hamilton, 1987; Cumming *et al.*, 1990). Moreover, poaching caused disruption of the social structure of the families and hierarchy of the breeding bulls. Poachers normally prefer large males with bigger tusks and when most of them are killed, adult females with relatively big tusks become their next target (Moss, 1988; Poole, 1989b; Barnes and Kapela, 1991).

Female African elephants live in stable maternal family led by the matriarch while males live solitary life or in temporally male herds (Moss, 1988; Moss, 1992). Poachers most often kill large, breeding bulls and cause disruption of mating competition such that young bulls get access to oestrous females and can have successful mating. This may have deleterious consequences on the general fitness of the entire population and hence the survival of the offspring when faced with environmental challenges such as drought.

Old female elephants are the main source of knowledge of their habitat and the death of the matriarchy usually results into disintegration of the whole family (McCombe *et al.*, 2001; Moss, 2001; Foley, 2002). Because of that, survival of the entire family is jeopardised as young elephants lack experience to lead the family and locate resources such as food and water during drought (McCombe *et al.*, 2001; Foley, 2002). For conditions where all adult females are killed, the remaining young elephants can reorganise into a family of unrelated individuals or get adopted by other bond family. In severe cases, some or all members of the affected family may die (Moss, 1988). Thus, an elephant family from a heavily poached elephant

population is likely to be composed of altered groups and probably genetically unrelated individuals (Foley, 2002).

Because of intensive poaching which threatened extinction of African elephant, all the elephants in Africa were put in Appendix 1 of the Convention on International Trade in Endangered Species of wild fauna and flora (CITES). CITES Appendix I provides for the highest conservation level such that any trade on the animal or its products requires a special permit from CITES. Thus, listing of the African elephants in Appendix I in 1989 was followed by the ban on international trade involving live elephant and any of their products especially ivory (Cumming *et al.*, 1990; Dublin, 1995).

The CITES ban was followed by a subsequent decline in poaching and ivory trade due to intensified protection and law enforcement. This resulted in a positive increase in elephant population in most range-states (Dublin, 1995). For example, studies done in Tarangire, and Mikumi National Parks in Tanzania and Addo National Park in South Africa have shown that population growth is significantly high in recovering populations following heavy poaching (Ereckson, 2001; Whitehouse and Hall-Martin, 2000; Foley, 2002). The results of these studies clearly indicate that given high level of conservation and food abundance, elephants have a very high population growth enough to recover from poaching effects.

Apart from the steps taken by CITES, there are reports of continuing poaching in different countries as well as existence of illegal ivory trade (Barnes *et al.*, 1999;

Martin and Stile, 2000). Moreover, the decision to drop elephant population from Zimbabwe, Namibia and Botswana to CITES Appendix II in 1997 and the one-day sale of ivory to Japan, has been associated with a significant increase in poaching in some African countries (Environmental Investigation Agency, 2000; Martin and Stile, 2000). Other major problems facing the African elephants in the entire region include, high human population growth, poverty, lack of arms control, lack of resources for conservation activities, under-trained manpower and habitat loss due to human settlement (Cumming *et al.*, 1990; Kangwana, 1995). These problems and other limitations such as ignorance facing conservation institutions in general and the emerging bush meat crisis really need immediate actions for the sustainable utilisation of wildlife in Africa (Barnett, 2000; Martin and Stile, 2000).

Good wildlife conservation strategies require accurate knowledge of different aspects of species under consideration. The conservation of African elephant has been facing a serious challenge due to the lack of knowledge on different factors affecting the elephant population, including poaching. For a long time, assessment of the impact of poaching on elephant population has based on the use of methods that estimate the size of elephant populations in different countries (Douglas-Hamilton, 1987; Barnes *et al.*, 1999). Some of the limitation of this approach has been its failure to provide information on the long-term effect of poaching on the socio-biological aspects of the elephants (Foley, 2002).

Other methods have therefore been employed to determine the effect of poaching on elephant population and these involved systematic study of the socio-biology and ecology of severely poached elephant populations (Balozi, 1989; Poole, 1989a;

Barnes and Kapela, 1991; Ereckson, 2001; Whitehouse and Hall-Martin, 2000; Foley, 2002). Studies done in Tarangire National Park (TNP) have shown that, the population was heavily affected by poaching as depicted by the sex and age structure, which are skewed towards females and young animals (Moss, 1990; Foley, 2002). These studies further showed that all male elephants in TNP were under the age of 30 years and only 4% of the females were above 30 years old, thus indicating that majority of the adult elephants were killed by poachers (Moss, 1990).

The above methods have also been used to provide behavioural and ecological information required for elephant conservation. However, there are other approaches that can be used to generate genetic information on elephant populations that cannot be obtained using the above techniques. These include immunological assays and protein electrophoretic procedures as well as the current most widely used DNA techniques. Immunological and protein (allozyme or general protein) electrophoresis have been used in the study of elephants and other wild animals with considerable success (Coetzee *et al.*, 1993; Avise, 1994). However, immunological tests often lack sufficient polymorphism enough to distinguish closely related species (Avise, 1994; Symondson, 2002). Furthermore, protein polymorphism may be lacking in some populations and may sometimes not be species specific. Thus, DNA techniques using either nuclear (nDNA) or mitochondria (mtDNA) DNA markers show great potential for use in conservation genetics and wildlife management (Balding, 1999).

Both nDNA and mtDNA have been used to quantify the effect of poaching on genetic diversity of the elephants in heavily poached and post-bottleneck elephant

populations (Nyakaana and Arctander, 1999; Whitehouse and Harley, 2001; Eggert *et al.*, 2002; Nyakaana *et al.*, 2002). The studies reported high genetic diversity among African elephants from different parts with exception of elephants in Addo and Kruger National Parks, which had low genetic diversity. The low genetic diversity in the two South African populations has been attributed to bottleneck effect due to poaching and confinement of elephants in small isolated areas leading to restricted gene flow (Whitehouse and Harley, 2001).

Although little work has been done on genetic diversity and population genetics of severely poached African elephants using DNA techniques, the DNA microsatellites isolated in this species have shown very high polymorphism (Nyakaana and Arctander, 1998, 1999; Eggert *et al.*, 2000; Comstock *et al.*, 2000). These DNA microsatellite markers can therefore, be utilised to study the population genetics of elephants and particularly kinship and parentage. This could complement the already available information aiming at understanding the effects of poaching on African elephants.

DNA microsatellite are simple tandem nucleotide repeats, located in both protein-coding and non-coding segments of the DNA (Armour *et al.*, 1999). They are hypervariable due to their relatively high rate of mutation and are widely spread throughout the eukaryotic genome. In addition, they are codominant and neutral markers whose analysis has been shown to have high potential for studying wildlife populations (Queller *et al.*, 1993; Chambers and MacAvoy, 2000). The use of DNA microsatellites together with the fast developing DNA techniques such as

Polymerase Chain Reaction (PCR) and sequencing provide powerful tools sufficient enough to identify an individual animal in natural population (Queller *et al.*, 1993; Woods *et al.*, 1999). The ability of DNA microsatellites to reveal varying polymorphic indices is critical in some studies, such as determination of genetic relatedness among closely related individuals and in parentage analyses (Queller *et al.*, 1993).

However, one of the limiting factors that has hindered comprehensive genetic studies in African elephants is associated to the difficulty in obtaining sufficient samples from free-ranging animals. This limitation has been attributed to the large body size, ecological factors, costs and permits required when handling of the elephants is inevitable (Khon and Wayne, 1997). Thus, other alternative sampling methods have been developed that enable to obtain elephant specific DNA non-invasively using faeces which can be obtained without handling the animals (Morin *et al.*, 1993; 1994; Constable *et al.*, 1995; Gerloff *et al.*, 1995; Khon and Wayne, 1997; Taberlet *et al.*, 1996; 1999). Using non-invasive approach, large number of faecal samples can be obtained simultaneously reducing the cost and undue stress imposed on animals that accrue from invasive sample collection procedures (Khon and Wayne, 1997; Wasser, *et al.*, 1997; Taberlet *et al.*, 1999).

This study was conducted in Tarangire National Park using non-invasive faecal sampling. The elephant population in TNP has been studied since 1993 and the whole population was sub-divided into three subpopulations (Foley, 2002) based on criteria described by Moss, (1988). The subpopulations were named as northern,

central and southern subpopulations depending on the specific parts of the park, which the animals use during the dry season. The northern subpopulation is the most studied and all females and males were identified using photographic files, ear markings and ear vein patterns (Foley, 2002).

The broad objective of this study was to determine the pedigree and level of genetic diversity among elephants in the northern subpopulation of TNP. The specific objectives were,

1. To determine the level of genetic diversity among elephants in the northern subpopulation as well as selected individuals in Central and Southern subpopulations of TNP
2. To establish the paternity within and between family groups.

It is therefore expected that the information obtained from this study will complement the available information on the genetic diversity of free-living elephants that were severely affected by poaching pressure. Further more, this study is expected to have enormous contribution on the efforts towards addressing the poaching crisis in African and the conservation of the African elephants in its broader perspective.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background information

The largest proportion of the elephant population in Tanzania is found in designated protected areas (PAs). Depending on the level of conservation and activities allowed in the PAs, they are subdivided into National Parks (NPs), Ngorongoro Conservation Area Authority (NCAA- for multiple land use), Game reserves (GR) and Game controlled areas (GCA) (MNRT, 1998). Whereas controlled utilisation through sustainable harvesting of resources is allowed in GR and GCA, harvesting of resources is prohibited in NPs and NCAA where the only utilisation allowed is by non-interference of the animals such as tourism, research and eco-tourism.

The German colonial government in 1905 established the first PA in Tanzania and at independence the country had a total of 13 PAs, of which 3 were NPs, NCAA and 9 GRs. After independence, the government increased the protected area to the current over 32% of its total range land (MNRT, 1998). These areas are occupied by a diverse spectrum of wild resources including threatened species such as endangered wild dogs, black rhinoceros, slender-snorted crocodile, chimpanzee, cheetah and the African elephant (MNRT, 1998).

However, the country faces a number of constrains which affect its current conservation efforts. Being one of the poorest countries in the world, it is faced with rampant poverty, which leads to fewer resources allocated for conservation activities and high financial constraints associated with budgetary limitations. As such, the

country is unable to fully fund wildlife conservation activities, research and other activities and hence making it dependent on donor support. The country also has a high population growth (over 3% per year) accompanied by encroachment of protected land for human settlement (MNRT, 1998). Furthermore, Tanzania is faced with high level of indiscriminate poaching both for subsistence and commercial purposes, institutional structure, legislation and policy related problems (Wasser, 1987; Cumming *et al.*, 1990; Tangley, 1997; MNRT, 1998; Malisa *et al.*, 2000; Vogel, 2001).

2.2.0 Geographical distribution of African Elephant

2.2.1 Identification of African elephants

Elephant belongs to the Kingdom animalia, Phylum chordata, Subphylum vertebrata and Class mammalia. It is classified in the Order proboscidea, which consist of animals with trunks or proboscis and are related to the animals in the order Sirenia and Hyracoidea. These three orders are sometimes put together in the superorder Panaengulata (Kleinschmidt *et al.*, 1986; Stanhope *et al.* 1998; Barriel *et al.*, 1999; Madsen *et al.*, 2001; Murphy *et al.*, 2001). The family elephantidae in which Africana elephants belong, consists of only two species of the surviving elephants. They include the Asian elephant (*Elepha maximus*) with about 29,000 to 40,000 elephants and the African elephant (*Loxodonta africana*) with about 300,000 to 600,000 animals (Barnes *et al.*, 1999; Barriel *et al.*, 1999; Houck *et al.*, 2001). Recent studies have actually shown that the African elephants are composed of two morphologically and potentially genetically distinct subspecies which should be regarded as different species composed of the forest elephants (*L. africana cyclotis*)

and the Savannah elephant (*L. africana africana*) (Tangley, 1997; Barriel *et al.*, 1999; Grubb *et al.*, 2000; Grove and Grubb, 2000; Roca *et al.*, 2001; Eggert *et al.*, 2002).

The elephants found in Tanzania belong to the Savannah elephants though some subgroups have been suggested and are thought to exist in Tanzania and other countries (Ereckson, 2001; Eggert *et al.*, 2002). Some of these include, Forest elephants in some parts like Udzungwa National Park (Wasser, S.K. personal communication, 2002) or a small subpopulation known as Masaai elephants (*L. Africana knochenhaueri).* However, no elephant population has been confirmed to belong to the Masaai or forest subspecies and thus, all the elephant populations in the country are regarded to be savannah elephants (*L. africana africana)* (Ereckson, 2001; Foley, 2002).

2.2 2 Distribution and status of African elephants

African elephants were originally found almost in all parts of the continent including North Africa and the Mediterranean coast during the European Middle Ages (Cumming *et al.*, 1990). However, elephants were exterminated in other parts and are currently restricted to the Sub-Saharan region occupying almost all types of habitat from savannah to rain forests, swamps, deserts, seashores and high mountains (Cumming *et al.*, 1990; Barnes *et al.*, 1999). Furthermore, the range and number of elephants have continued to decline towards the end of the last century due to poaching escalated by demand for ivory and its association with civil war in some range-states. In addition, this decline is associated with extensive desertification and

rapid growth of human population leading to conflicts for land use (Cumming *et al.*, 1990; Dudley *et al.*, 2001). Apart from that, ivory trade is taken to be a single factor that has contributed to the reduction of the population of elephants in Africa (Cumming *et al.*, 1990)

Following a decline of elephant population in many range-states between 1979 and 1980s, the African elephant was listed in Appendix I of the Convention on International Trade in Endangered Species (CITES) and the trade on ivory was banned in 1989 (Dublin, 1995). This is because CITES Appendix I contains all animals that are at high risk of extinction and hence all international trade on the live animals or their products for such species is strictly regulated by CITES. For that reason, all reported ivory trade and poaching taking place in different countries are illegal and need to be stopped by all countries which are member states of CITES (Dublin, 1995; Martin, 2000)

The decline in elephant population between 1979 and late 1980s was caused by widespread poaching resulting from the rapid increase of ivory trade and good price of ivory on the international market (Douglas-Hamilton, 1987). During that single decade, there was a tremendous decline of the number of elephants from 1.3 million to about 600,000. The rapid increase of poaching was also said to be associated with civil wars in many African countries that enabled poachers to get access to automatic weapons (Douglas-Hamilton, 1987; Cumming *et al.*, 1990). Likewise, there was an association between the sudden drop of petrol prices followed by global financial

instability in the early 1970s and the rapid increase of the ivory trade (Cumming *et al.*, 1990).

The severity of poaching was different among different countries in Africa. For example, countries such as Botswana, Zimbabwe, Namibia and South African were less affected by poaching while Tanzania, Kenya, Zambia, Democratic Republic of Congo (DRC) and Congo were severely affected (Douglas-Hamilton 1987). It was further reported that Uganda lost about 83% of its elephant population. The difference on the level of poaching that occurred among African countries has been manifested on the different elephant conservation measures taken by each individual country (Feral, 2000). While some countries require continued ban of ivory trade to allow their elephant population to recover, others want their elephants to be re-listed in Appendix II so that they can sell their big stock of ivory (CITES, 2000).

This lack of a continent-wide agreement on the conservation measures required for the African elephant is among the major limitations on the implementation of CITES decisions. Because of that, poaching and illegal ivory trade has been recently reported in some African countries despite the CITES ban (Martin, 2000). Thus, the elephant population in African is still facing the risk of extinction and deliberate continent wide conservation measures are urgently needed.

2.2.3 Elephants population in Tanzania

Elephants are among the wild animals found in most of the protected areas in Tanzania. They are widely distributed from South to Northern woodlands as well as some areas of the Western regions. The total elephant population in the country is estimated at 91,690 with northern and southern parts of the country having the largest number of elephants (Barnes *et al.*, 1999). However, it has been reported by Barnes *et al.* (1999) that, elephants are already extinct in some parts of the country such as Karagwe while Selous GR, Ruaha, Serengeti, Tarangire and Katavi NPs have the highest elephant population in the country. These are among the few protected areas in Africa with a large number of readily visible elephants especially during the dry season.

According to Douglas-Hamilton (1987), Tanzania lost about 53 % of its elephant during the period between 1977 and 1987. It was further established that the estimated number of elephant from selected parts of the country depicted a decrease from 184,872 to 87,088 elephants during the same period (Table. 2.1). The data also show that, the losses were very high in unprotected areas of Arusha, Tabora and Kilombero, which lost about 80% of the total population as compared to protected areas which lost about 49% of the elephants. The elephant population trends in selected areas of Tanzania for a period of over 20 years from 1977 to 1998 is summarised in Table 2.1.

Table 2.1: Estimated number of elephants from selected areas of Tanzania.

Location	1977	1987	1998	%Change (77-87)	%Change (87-98)	Total change
Unprotected areas						
Tabora**	8,399	1,958	1,709	-77%	-13%	-79%
Arusha***	16,660	2,146	-	-87%	-	
Kilombero	5,848	2,230	1,903	-62%	-15%	-68%
Subtotal	30,907	6,334	3,612	-80%	-14%	-75%
Protected areas						
Selous GR	109,000	55,000	31,735	-50%	-42%	-71%
Ruaha NP, Rungwa and Kisingo GR	43,682	21,986	19,111	-50%	-13%	-56%
Serengeti NP	3,008	395	2015	-87%	+510%	-33%
Manyara NP	453	180	-	-60%	-	-
Tarangire NP	3000	3000	1,814	0%	-40%	-40%
Mkomazi GR	667	193	346	-71%	+179%	-48%
Sub-Total	159,813	80,745	55,021	-49%	-32%	-66%
Total	184,872	87,088	58,633	-53%	-33%	-68%

***Tabora includes Gombe, Inyonga, Kigozi, Tabora South and Ugunda and Arusha areas include Endule, Hanang', Loliondo, Marang Forest, Masai steppe and Yaida.
Source: Douglas-Hamilton (1987), Cumming *et al.* (1990) and Barnes *et al.*, (1999).

2.2.4 Elephants population in Tarangire National Park

Although the elephant population in TNP was severely affected by poaching in the last two decades, there has been no current report of poaching in the Park (Foley, 2002). Due to lack of harassment by poachers like in the past, elephants in the Park and particularly in the Northern part have become relatively tame and allow vehicles to approach them up to within 20 metres (Foley, 2002). Together with short vegetation covers that allow visibility over a long distance, the above features make Tarangire elephant population one of the best sample for studying the effects of past poaching on various parameters of elephants' socio-biology (Foley *et al.*, 2001; Foley, 2002).

Different studies done on the population dynamics of elephant in TNP have shown that there has been a stable elephant population in the Park (Table 2.1 and 2.2). This would imply that elephant population in TNP was not severely affected by poaching (Douglas-Hamilton, 1987, Cumming *et al.*, 1990, Barnes *et al.*, 1999). However, studies done on the age, sex structure and social behaviour have actually shown that elephants in TNP were severely affected by poaching like other elephant populations in Mikumi, Ruaha and Queen Elizabeth NPs (Moss, 1990; Poole, 1989a; Balozi, 1989; Barnes and Kapela, 1991; Ereckson, 2001; Foley *et al.*, 2001; Foley, 2002). The high and stable number of elephants reported in Tarangire was said to be due to immigration of elephants from surrounding areas into the park, which was found to be relatively safe because of frequent patrols done by Park Rangers (Foley, 2002). The trends of the estimated population of elephant in TNP from 1958 to 2001 are summarised in Table 2.2 below.

Table 2.2: Trends of the estimated number of elephants in TNP from 1958 to 2001.

Year of survey	Population size*	Author
1958-1961	550	Lamprey, (1993), (1964)**
1978	1342 484	Douglas-Hamilton** (Unpublished)
1980	2891	Ecosystem Ltd (1980)**
1987	5683 ± 1652	SEMP (1987)**
1987	3000	Douglas-Hamilton (1987), Cumming *et al.* (1990)
1988	2420 ± 872	SEMP (1988)**
1991	1992	TWCM (1992)**
1998	3000	Barnes et al. (1999)
2000	2000	TAWIRI (2000)**
2001	2300	TAWIRI (2001)**

** Obtained from Foley (2002).

* The figures represent estimated number of elephants ± standard error of the means.

2.3.0 Social relationship among African elephants

2.3.1 The family

The basic social unit of elephants is the family, which consists of 6-12 and sometimes up to 20 animals with variation in family size among populations, season of year as well as the extent of poaching that occurred in the population (Moss, 1988, 2001; Poole, 1989a; Foley, 2002). The Savannah elephant family unit is composed of the big female, her infants, close relatives and their infants. The family is usually led by a matriarch, which is the oldest and most experienced member of the herd as well as the source of all the knowledge required by the family especially during scarcity of resources (Moss, 1988; McComb *et al.*, 2001; Foley, 2002). However, forest elephants live in very small groups with the largest family containing mean group size of 3 individuals (Tangley, 1997; Theuerkauf, *et al.*, 2000).

The family size is normally maintained under favourable conditions and splits in case of food shortage, adverse environmental conditions or when it becomes too big to

manage as a single unit (Western and Lindsay, 1984; Moss, 1988). Due to behavioural indicators displayed by family members, different parameters are usually used to categorise an individual elephant as a member of a given family. Family members seem to spend more time together and act in a coordinated manner like feeding, drinking, travelling or resting at the same time while taking all the commands from only the matriarch (Moss, 1988).

Furthermore, members of a family maintain very close contact by various means and the biggest indicator of relatedness among member of the family is thought to be the strength of greeting ceremony whose nature and intensity depends on relatedness among individuals and time they have been separated. For closely related individuals separated for a relatively long time, greeting ceremony is accompanied by greater energy and excitement together with activities such as running towards each other, different types and levels of touching, rumbling, trumpeting, screaming, different types of movements, urination and defecation (Moss, 1988).

Due to the importance of the matriarch, its death causes destabilisation of the family and infants less than 6 years old usually die during the transition period before the next old member takes over the leadership (Moss and Poole, 1984; Lee and Moss, 1986; Moss, 1988, 1992; McComb *et al.*, 2001). Family members are believed to have a high degree of maternal relatedness though the exact relatedness and paternity of individual has been difficult to reveal using behavioural studies alone as in other wild animals such as primates (Morin *et al.*, 1993, 1994; Constable, *et al.*, 1995, 2001). Thus, the relationship among members of the family might be the reason for

the close bondage that exists among all family members, established early in the life of an individual animal to ensure their survival (Moss, 1988; Foley, 2002).

2.3.2. Bond groups

The bond groups are composed of elephant families that interact more frequently than any other families in the population and have high level of association patterns. Bond groups spend more time together and the families show unique behaviour such as coordinated activities led by the dominant female (Moss, 1988). The families also show high intensity of greeting ceremony after separation as opposed to unrelated or distantly related families (Moss, 1988). The formation of bond groups is thought to be through fission of large families and number of families that form a bond group vary from one population to another (Moss, 1988; Foley, 2002). It has however been observed that bond groups may be absent in populations that have been constantly harassed by poachers such that elephants move in big herds probably for defence reasons (Poole, 1989a; Foley, 2002).

2.3.3 Clan and other levels of organisation

The social organization of elephants has been described by Moss (1988) to exhibit a circle of relationship where animals at the centre form the family followed by the bond groups. Outside the central part of the circle, there are elephant families that use the same dry season range, interact more frequently than others though they show a high degree of aggression than members of the same family or bond group. The different families in this category, might form big aggregations depending on food availability but reveal no close bond with one another and make no greeting

ceremony. Such families form a higher level of social organisation known as a clan. Clan size differs according to habitat type and geographic location. Different clans that share the same dry and wet season ranges form a subpopulation whereas different subpopulations which may have overlapping wet season range and bulls form the whole population in a given area for the entire population (Moss, 1988).

The system of dry season home range disintegrates during the rain season when food is plenty, such that different families join together in large herds and move through different parts of the ecosystem. In such a herd, members of different families meet, interact and eventually determine or establish their social rank with some individuals establishing their dominance (Moss, 1988). It is because of aggregations in big herds, elephants cause more damages upon crop raiding during the rain season (Kangwana, 1995). This is an important aspect that should be considered when planning conservation activities in areas with high human-elephant conflicts.

2.3.4 Social relationship with males

The relationship within bulls themselves and also between bulls and female elephants depends on their physiological status as well as the season of the year. Females interact with bulls depending on their reproductive state, age of the bull, the kin relationship between the bull and the family as well as the physiological state of the bull (Moss, 1988; Poole, 1987, 1989b; 1989c). Young male up to 20 years of age may be tolerated by the family upon joining it while older males spend less time with females during the dry season. During this period, female elephants show no interest to males under normal conditions unless in heat. Adult and experienced females in

oestrus usually avoid young males aged 20-30 years but attract, make consort as well as allow mating with older bulls especially those in musth (Poole, 1987, 1989b, 1989c). During the rain season however, female and male elephant interact more frequently and are found together in big herds with both old and young bulls.

2.3.5 Communication

Communication among elephants is important for their survival and take place among related elephants or even the entire population. It has been shown that elephants do respond to sounds from very long distances, which are not audible to human in that area (Moss, 1988). In communicating to each other, elephants use a variety of methods such as visual displays, touch, chemical secretion and many different types of sounds (Moss, 1988; Rasmussen and Schulte, 1998; Poole, 1999; Lagbauer, 2000; Rasmussen and Wittemyer, 2001). Chemicals secreted by elephants in urine and other body fluid play a very important role in kin and individual recognition such that there are speculations concerning use of chemicals from urine by bull for kin recognition and hence avoiding inbreeding (Lagbauer, 2000). Furthermore, elephants of different ages produce different types of sound for various purposes including audible sound such as trumpeting, roars, rumbles and squeaks as well as infrasound which is a very low frequency sound inaudible to humans (Moss, 1988, Poole *et al.*, 1988; Garstang *et al.*, 1995; Poole, 1999; Lagbauer, 2000). However, elephant communication via sounds and especially by means of infrasonic sounds, exhibit a marked diurnal variation with peak 1-2 hours after sunset (Garstang *et al.*, 1995).

2.4.0 Reproduction in elephants

2.4.1 Puberty and mating in males

Bull calves live in their maternal families until when they reach 11-14 years of age, the time at which male calf start to attain reproductive maturity and puberty. After reaching maturity, male elephants leave their maternal family and live the rest of their entire lives either individually or in temporally bachelor herds (Poole, 1987; Moss, 1988). It is only during mating or during the rain season when male elephants are found among families with or without oestrus females in large aggregations of elephants (Poole, 1987, 1989b; Foley, 2002). Studies on the association pattern among elephants have shown that bulls in musth associate more with females during rain season than non-musth bulls (Poole, 1987). Furthermore, these studies have shown that young males hardly mate successfully in the presence of big musth-bulls over 30 years. These musth-bulls monopolise most of the matings and attain the highest successful mating in the population (Moss, 1983, 1988; Poole, 1987, 1989b, 1989c; Foley, 2002).

2.4.2 Puberty and reproduction performance in females

Female elephant reach puberty earlier than males and are reproductively mature between the ages of 7 to 13 years with the mean age at maturity varying among different populations (Moss, 1988, 2001; Whitehouse and Hall-Martin, 2000; Foley, 2002). Upon reaching reproductive maturity, adult females elephants experience cyclic ovarian activity that is manifested as oestrus behaviour (Moss, 1983). The oestrous cycle of free-ranging African elephants is 16 week and they experience heat periods for 4-6 days (Moss, 1983). Reproductive activities in female elephants

continue until in their late 50s when they exhibit a marked decrease and eventually cease before reaching the age of 60 years (Moss, 1988, 2001).

Female elephants can potentially come in oestrus throughout the year though it has been shown that majority of the elephants come in oestrus during and just after the rain season (Moss, 1983). Following abundance of food during the rain season, females synchronise their breeding by coming in heat within the same period of time and the synchronisation is thought to be mediated by visual and chemical stimuli (Rasmussen and Schulte, 1998; Rasmussen and Wittemyer, 2001). Most of the matings occur during this time and female elephants have been shown to exercise choice for the males to mate with (Poole, 1989b). Moreover, it has been shown that only adult females can seek consort and mate with musth bulls since young females cannot utilise better the guiding habit of the bulls in musth and this makes them vulnerable to mate with relatively younger and non-musth bulls (Moss, 1983, 1988; Poole, 1987, 1989b; Foley, 2002). Thus, the ability of adult females with sufficient experience to choose and mate with the best musth-bulls has been thought to increase the fitness of their offspring as shown by low infant mortality in females over 25 years old (Poole, 1989b).

However, reproductive activities of female elephants of all age classes are normally affected by the season of the year and therefore food availability. As such, elephants stop cycling and therefore breeding stops during severe drought when food becomes scarce (Moss, 1988; Foley, 2002). Foley *et al.* (2001) found that during dry season, female elephants have markedly high level of cortisol and low level of progesterone,

which indicates that breeding activities are depressed as a result of environmental stress.

2.4.3 Musth in male elephants

A phenomenon of musth, which resembles rutting in some ungulates, was first described in Asian elephant and later in African elephants as well (Poole and Moss, 1981; Poole *et al.*, 1984; Poole, 1987). It is an annually cyclic process, which occurs in reproductively mature male elephants from the age of 20-30 years (Poole *et al.*, 1984; Poole, 1987; Moss 1988). Musth in male elephants is associated with changes in behavioural as well as hormonal levels. Behavioural characteristics of a bull in musth include a distinctive posture, swollen and secreting temporal glands, dribbling of strong smelling urine and green penis. Other behavioural changes include increased aggressive nature, increased association with female families and production of low frequency sounds known as musth rumble (Poole, 1981, 1987, 1999; Poole *et al.*, 1984; Moss, 1988). The duration of musth has been shown to vary with age and body size where young males (20-30 years old) experience a recurring musth for a few days up to a week while older bull of over 40 years old experience a prolonged musth of between 2 to 4 months (Poole, 1987, 1999).

Musth is a costly and energy-demanding process since the bull in musth spend less time on feeding and instead searches and attempts to access or guard oestrus females. The social rank of the bull changes during musth such that a bull in musth becomes dominant to all non-musth bulls with some few exceptions (Poole, 1987). The interaction among bulls in musth is usually an aggressive one and depends on the

body size and body condition as well as the stage of musth. It has been observed that a big bull at the end of musth will always be submissive to a young bull just at the onset of musth (Poole, 1987,1999). Unlike oestrus in female elephants, the period of musth is asynchronous and it is accompanied by elevated levels of testosterone in blood and urine (Howard *et al.* 1984; Poole *et al.*, 1984; Poole, 1987; Rasmussen and Schulte, 1998; Rasmussen and Wittemyer, 2001).

2.4.4 Gestation and calving

Reproduction in female elephants is not strictly seasonal though in most cases calving has been reported to occur before and during the rain season (Moss, 1988). The gestation period of the elephant is 22 months and the intercalving interval ranges between 2.5 and 4 years but it can extend up to 9 years depending on the food availability. However, the intercalving interval reported differs among different populations (Moss, 2001; Whitehouse and Hall-Martin, 2000; Foley, 2002). Intercalving interval, age at first conception and the age of senescence-induced fertility decline have been pointed out as the best measure of reproduction rate in free ranging female elephants (Moss, 1988; Foley, 2002).

2.4.5 Lactation and weaning

In African elephants infants continue to suckle until another calf is born. Males have been shown to suckle more frequently than female calves and have extended dependence on mother's milk (Lee and Moss, 1986). Weaning in elephants is a gradual process, starting in the first year of life and may continue up to the tenth year but in most cases complete weaning occurs when another sibling is born. Normally,

the calves are fully weaned at the age of 5-6 years with females weaned earlier than males (Dublin, 1983; Lee and Moss, 1986; Moss, 1988). This prolonged dependency period is vital to the elephant as it is one of the means that ensure survival of the offspring.

2.4.6 Twinning and infertility

Twin births have been reported in elephants and it has been observed to be rare in free ranging African elephants (Moss, 1988; Moss, 1992; Foley, 2002). However, Foley, (2002) reported the highest rate of 5% twin births in Tarangire National Park, with most of them involving closely related females. On the other hand, infertility has been recorded in African elephants (Moss, 1992; Whitehouse and Hall-Martin, 2000; Foley, 2002), though the reproductive performance of elephants under good nutrition has been found to be very efficient. This high reproductive performance of elephants together with low level of infertility are thought to be the most important factor in recovering elephant populations following heavy poaching (Foley, 2002) and in post-bottleneck populations (Whitehouse and Hall-Martin, 2000).

2.4.7 Allomothering and calf survivorship

In an elephant family, calves are not only nursed by their mothers, but also by other females within the family. This strategy which is called allomothering, has been reported in species such dolphin and African elephants (Dublin, 1983; Moss and Poole, 1984; Lee and Moss, 1986; Moss, 1988; Parson, 2001). This behaviour has been described as an important strategy to ensure the survival of the calves and especially when the mother is incapable of suckling the calf or in cases of death of

the mother. Calf survival has been shown to be high in big families with many allomothers than small families (Lee and Moss, 1986). This is because the calves in big family obtain more security from other family members and this has been shown to increase the survivor rate of infants.

2.5.0 Genetic polymorphism in natural populations

In all living organisms, the inheritable genetic materials are contained and stored in DNA with exception of some RNA viruses. Prokaryotes such as bacteria have simple, circular DNA while eukaryotes (plants and animals) have very long and linear DNA molecules (Alberts *et al.*, 1989). The DNA in eukaryotic cells is more complex and made up of both protein coding and non-coding regions. Nearly all eukayotic cells' DNA is located in the nucleus, known as nuclear DNA (nDNA) with exception of organelle DNA found in mitochondria (mtDNA) of all species and chloroplasts (cpDNA) of photosynthetic plants. Each nDNA molecule is packed in a separate chromosome and the total genetic material in all chromosomes and organelles of an organism constitutes its genome. Thus, all sources of variations within and between different species are believed to be due to variations at both nDNA and mtDNA (Alberts *et al.*, 1989).

Polymorphism refers to variation within species and it is considered to be independent of ontogeny and sex of an individual (Wiens, 1999). It has further been defined as a condition under which a given DNA locus has more than one allele and at least 5% of these alleles in a population are different from the most common ones (Goldstein and Schlotterer, 1999). The variation is genetically based and hence

heritable. This variation can be discrete (qualitative) or continuous (quantitative). The variation in quantitative traits has been widely used in quantitative genetic studies whereas qualitative traits are the basis of population genetics and related studies (Falconer and Mackay, 1997).

Polymorphism within species is abundant in all phenotypic and genotypic traits including morphology, proteins such as allozymes and DNA sequences. Major forces that cause genetic variation in natural systems include mutation and recombination, genetic drift, selection and migration (Falconer and Mackay, 1997). Thus, studying polymorphism in natural populations is a very important and critical component of conservation genetics.

A variety of methods have been used to study polymorphism among individuals at different levels of social organization in natural systems. The methods used range from systematic study of the organisms using different methods such as those used in systematics and comparative biology to the current genetic approaches based on genetic polymorphism at the DNA level.

2.5.1. Non-DNA based methods

Traditionally, external and internal phenotypic characteristics have been used for a long time to classify organisms to different taxonomic levels. The methods are based on the comparison of different features within and between species. Example of such features employed in evolution, comparative biology and phylogenetic studies include anatomical structure such as limbs, beaks and wings in birds, feathers and

coat colours as well as ecological, reproductive and feeding behaviour (Darwin, 1859; Huey *et al.*, 2000). Furthermore, attempts to study single gene variations were undertaken by studying morphology and behaviour of mutants in comparison to wild individual of the same species (May, 1992). However, comparative studies over a broad geographical range are difficult because of many factors, which hinder the study of individual population over a sufficiently long time (Fleischer, 1996; Maddison, 1996).

Protein electrophoresis (PEP for allozymes and general proteins) is a technique used to indirectly measure DNA variation among different individuals in a population. The method is based on the difference in distance migrated by proteins when a protein solution on a gel is subjected to an electric current. The migration is due to the different charges of some amino acids, size and shape of three-dimensional structure of a protein. This technique utilises the procedure of separating protein solutions by electrophoresis followed by histochemical staining which reveal different allele depending on the speed of the proteins and gives three alternative forms (fast, medium or slow alleles) (May, 1992). The alleles obtained usually offer a relatively cheap and fast method for analysing single locus variation in the population (May, 1992). Thus, different biochemical markers used in PEP are indirect indicators of genetic differences on the DNA, which codes for those proteins (Eding and Laval, 1999).

Biochemical markers based on PEP technique have been widely used in studies involving natural populations based on allele frequencies at different loci such as

population analysis, systematics, parentage analysis and hybrid identification (May, 1992; Coltman *et al.*, 1999). Studies utilising allozymes have been done in a number of species such as reptiles, birds and vertebrates where the results obtained have been able to provide useful information on evolution and genetic diversity of different species (Kocher *et al.*, 1989; May, 1992; Coetzee *et al.*, 1993). Due to fast development in molecular biology techniques, biochemical methods are being continuously replaced by the most recent DNA-based techniques that directly measure DNA variation (May, 1992; Maddison, 1996; Coltman *et al.*, 1999).

Other non-DNA based methods have also been used in studying polymorphism in natural populations. These methods include blood typing, karyotyping and immunological assays. For a long time, blood typing using different systems such as ABO, MN and other blood groups in human, have been widely used in population studies including relatedness and paternity analyses (Falconer and Mackay, 1997). ABO system of blood typing is based on the presence of surface antigens on the red blood cells, which bind to specific antibodies and cause alterations of the cell structure leading to clamping or agglutination. Both blood typing and immunological assays depend on the reaction between antigens and specific antibodies, thus, they lack the power to resolve differences among closely related groups such as breeds due to cross reactivity of the antibodies.

2.5.2 DNA-based methods

Different techniques have been developed to assess the extent of polymorphism at the gene level using different DNA markers. Specific DNA markers are used in most

studies to measure genetic distances based on differences in allele frequencies among different populations. The DNA polymorphic markers commonly used in genetic studies include random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Variable number of Tandem repeats (VNTRs) and single nucleotide polymorphism-SNP or DNA-chips (Southern, 1975; William *et al.*, 1990; Avise, 1994; Eding and Laval, 1999). VNTRs are made of groups of markers among which are satellites, minisatellites and microsatellite markers (Litt and Luty, 1989; Tauz, 1989; Weber and May, 1989; Beckmann and Waber, 1992). Thus, determination of genetic variation using polymorphic DNA markers provides the best objective information in situations where other supporting data is missing or incomplete.

The use of DNA based methods has experienced a rapid development due to the benefit accrued from the fast developing molecular biology techniques. Examples of such techniques include DNA cloning, genetic engineering, nucleic acid hybridisation and rapid sequencing of DNA fragments. Cloning of DNA has for along time depended on *in vivo* methods whereby an entire genome is digested with restriction enzyme, inserted in a vector and amplified using a specific bacteria host such that fragments of interest can be obtained in numerous copies contained in bacterial genome (Alberts *et al.*, 1989). Following discovery of *in vitro* amplification of DNA fragment by polymerase chain reaction (PCR), DNA cloning using PCR has contributed enormously as an important tool for studies that require amplification of small amounts of DNA (Mullis and Falcon, 1987; Saiki *et al.*, 1988). PCR has made it possible to amplify small amounts of DNA from a single sperm, museum

specimen, faeces, hair and other sources with very little template DNA (Taberlate *et al.*, 1996; Khon and Wayne, 1997).

Furthermore, genetic engineering that involve altering of DNA sequence to make a modified version of the gene, which are re-inserted back into the cell/organism has contributed extensively to the fast developing field of biotechnology (Alberts *et al.*, 1989; Li and Graur, 1991). Examples of the areas that have benefited from both genetic engineering and DNA cloning include plant and animal biotechnology such as production of genetically modified products of both plants and animal origin (Eding and Laval, 1999).

On the other hand, the development of techniques for rapid sequencing of any DNA segment of interested is another area of biotechnology that has been useful in many studies that measure actual variation in the DNA and hence analysis of DNA structure and function (Sanger and Coulson, 1975; Maxam and Gilbert, 1977; Sanger *et al.*, 1977). Combination of sequencing and other methods such as mtDNA, RFLP and microsatellite analyses have been useful in the construction of molecular phylogenies (Kocher *et al.*, 1989). However, the pre-supposed distance between taxa affects the selection criteria of the genomic region to be sequenced such that low genetic distance between individuals may require sequencing of very long DNA strands (Meghen *et al.*, 1994; Fleischer, 1996). Sequencing is generally considered to be laborious and expensive, but the development of automated sequencing is likely to make it a normal laboratory procedure.

2.5.2.1 Mitochondria DNA (mtDNA)

Mitochondria DNA (mtDNA) is a relatively small, haploid and simple organelle DNA. It has circular structure in almost all organisms except some algae and protozoa and it is present in more than one copy in a single mitochondrion (Alberts *et al.*, 1989). The size and functional parts of mtDNA differs in different organisms. For example, mammals have the smallest mtDNA (about 16.5kb) without introns while plants have the largest mtDNA (150-2500kb) and some segments in plants have no specific functions (Alberts *et al.*, 1989). Like cpDNA, mtDNA carry out its own DNA replication, transcription and protein synthesis. Due to maternal mode of inheritance and lack or recombination, mtDNA has been utilised to provide information about female genealogy and the assessment of the matriarchal lineage in the population (Avise, 1994).

The use of PCR and RFLP on mtDNA markers has been utilised in establishing the pattern of genetic divergence between and among species as well as in species identification (Avise, 1994; Wasser *et al.*, 1997; Malisa *et al.*, 2000; Clarke *et al.*, 2001). Using mtDNA, Georgiadis *et al.* (1994) were able to show that elephants in Africa have a complex population structure and isolation by distance exist at region levels between Eastern and Southern Africa elephant populations. Moreover, when both mtDNA and RFLP are combined with sequencing and nuclear DNA markers such as microsatellites, they provide very high polymorphism suitable for studies of population genetics and evolution history among different species (Kocher *et al.*, 1989; Clarke *et al.*, 2001; Eggert *et al.*, 2002; Nyakaana *et al.*, 2002)

2.5.2.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based technique developed by William *et al.* (1990). The technique uses single short primers (about 10bp) of arbitrary sequences to amplify nuclear DNA by polymerase chain reaction (PCR). The amplification is based on the random binding of the primers on any genomic DNA region flanked by a pair of priming sites in the opposite orientation within 5kb of each other (Welsh and McClelland, 1990; William *et al.*, 1990, 1993; Perez *et al.*, 1998; Ratnayeke *et al.*, 2002). By using low annealing temperature, the technique allows amplification of fragments of different sizes from the entire genome and individuals are scored for presence or absence of a particular band through comparison with a molecular size standard for RAPD markers (Ratnayeke *et al.*, 2002). The absence of the band may be caused by failure to prime a site in some individuals due to differences in nucleotide sequences, or insertions/deletions between two priming sites.

RAPD has been extensively used for genetic characterisation of a wide range of organisms, taxonomy, paternity analyses and mapping (Perez *et al.*, 1998). The method has a capacity of displaying genetic variation and requires no prior knowledge of DNA sequence to be analysed. Furthermore, RAPD has been found to be less expensive, can be used with limited amounts of DNA, is suitable for work on anonymous genome and as opposed to other markers, it can detect polymorphism in any kind of sequences (William *et al.*, 1990, 1993; Perez, *et al.*, 1998). Due to the ability to amplify DNA segments of unknown sequence, RAPD has been used in isolation and studies of microsatellite polymorphism (Orti *et al.*, 1997).

However, the method has not received equivocal acceptance because it is not yet known on how genetic variation is determined and fragments have less reproducibility while sensitive to changes of stringency (Perez *et al.*, 1998). This causes difficulty in reconstruction of evolution histories; and the accuracy of the data is not guaranteed. Moreover, RAPD is a dominant marker and hence does not differentiate heterozygotes and homozygotes leading to reduced content of information (Grosberg, 1996).

2.5.2.3 Restriction Fragment Length Polymorphism (RFLP) and AFLP

RFLP was the first DNA polymorphism technique to be widely used for genome characterisation (Southern, 1975). The application of RFLP depends on the use of restriction enzymes to cleave the entire DNA at specific points known as restriction sites and the subsequent analysis of the fragments using specific DNA probes (Li and Graur, 1991). The method is based on mutation and substitution of nucleotide bases that removes or adds a restriction site, and hence low rate of mutation renders the method unable to resolve differences between closely related groups (Aquadro *et al.*, 1992; Fleischer, 1996).

The banding patterns produced by restriction fragments can be used to study genetic distance, population variation, gene flow, effective population size, pedigree and patterns of biogeography. With application of PCR, the use of RFLP has been improved by using restriction enzymes to cleave specifically amplified DNA segment and then analysing the amplified fragments for length polymorphism (Malisa *et al.*, 2000; Clarke, *et al.*, 2001). This reduces the number of restriction

fragments and hence increases the ability to resolve difference between closely related groups.

However, RFLP has some limitations because it requires large quantity of good quality DNA and non-PCR/RFLP is labour intensive due to hybridisation step that is difficult to automate. Moreover, the informativeness of RFLP markers is low compared to other DNA based methods.

2.5.2.4 Variable Number of Tandem Repeats (VNTRs)

The term variable number of tandem repeats (VNTR), was first introduced by Nakamura *et al.*, (1987) for microsatellites but it is currently used to refer to a group of three different genomic DNA markers (Chambers and MacAvoy, 2000). These include satellite, minisatellite and microsatellite depending on the size of the repeat array (Armour *et al.*, 1999; Chambers and MacAvoy, 2000). The nomenclature of these markers has been recently reviewed by Chambers and MacAvoy (2000) who proposes that VNTRs be divided into three categories, which are satellites (100bp repeats and above), minisatellite (10-100bp repeats) and microsatellites (2-6bp repeats). According to the authors above, uniform runs of a single nucleotide are known as mononucleotide tracts and are neither considered as microsatellites nor VNTRs. However, the gap between microsatellites and minisatellites (i.e. 7-10bp repeats) has been left with such repeats unnamed, because they have not been isolated and hence not applied in any molecular studies.

Satellites can occupy a big part of the genome and the individual array can be as big as 5Mb (Armour *et al.*, 1999). According to a review by Armour *et al.*, (1999), satellites are used occasionally for genotyping of individuals in a study population. They can be typed by Southern blot hybridisation after pulse-field gel electrophoresis to measure the total array size; restriction digestion or using PCR primers that detect locus-specific repeat unit variants.

Minisatellites have arrays of sizes between 0.5-30kb with number of loci per genome in the range of hundreds or thousands and hence considered to have the highest polymorphism among all VNTRs markers (Armour *et al.*, 1999; Balding, 1999; Beaumont and Bruford, 1999). Minisatellites can be typed using multi-locus fingerprinting by hybridisation employing low-stringency probes or single-locus genotyping that utilises high stringency probes (Balding, 1999; Bruford *et al.*, 1992). Despite high polymorphism displayed by minisatellites, their use is currently lagging behind that of microsatellites due to difficulty in automation and other limitation associated with minisatellite scoring (Balding, 1999; Beaumont and Bruford, 1999).

Microsatellites are DNA sequences with one sequence motif of up to six bases long and have been categorised as dinucleotide (for example AT), trinucleotide (CAC)- or tetra-nucleotide (TTTA), based on the number of repeating nucleotide units provided the array size is greater than 8bp (Chambers and MacAvoy, 2000). Microsatellites described seem to exist into six different classes or families based on the arrangements of the repeating units (Table 2.3). These include pure (same nucleotides repeat units), interrupted pure (different nucleotides occur between the

same repeat units), compound (repeats of different units), interrupted compound, complex and interrupted complex type of microsatellites. Complex microsatellites are made of different types of repeat units such as di- and tetra-nucleotide units and when these units are interrupted by other different nucleotides in between, interrupted complex microsatellites are obtained (Chambers and MacAvoy, 2000).

Table 2.3: Examples of the six classes of microsatellites.

Class	Locus name	Sequence
Pure	Spun 10	$-(AC)_{14}-$
Interrupted pure	Spun 210	$-TA-(CA)_{4}-TA-(CA)_{7}-$
Compound	Spun 14	$-(CT)_{22}-(CA)_{6}-$
Interrupted compound	*Ort*FCB24	$-(AC)_{14}-AG-AA-(AG)_{12}-$
Complex	FGA	$-(TTTC)_{3-4}-(T)_{6}-(CT)_{0-1}-(CYKY)_{n}-CTCC-(TTCC)_{2-4}$
Interrupted complex	FGA	Some FGA

Source: Chambers and MacAvoy (2000).

Following the discovery of microsatellites by Tauz (1989), Weber and May (1989) and Beckmann and Waber (1992), it has been shown that microsatellite polymorphism may perhaps be the most useful technique in resolving differences between closely related groups. Moreover, microsatellites are abundant and uniformly distributed in different eukaryotic genome occupying both coding and non-coding regions. They have high level of mutation rate, are neutral and codominant markers (Tauz, 1989; Schlotterer and Wiehe, 1999). In addition, microsatellite polymorphism can be described numerically, and therefore, it enables computerised data handling and analysis using small amounts of DNA (Balding,

1999; Beaumont and Bruford, 1999). However, application of microsatellite markers requires prior knowledge of the DNA sequence of the segment to be amplified for the design of the primers and many loci have to be typed to obtain the desired information (Fleischer, 1996).

It has been further established that microsatellites loci are longer and more common in vertebrates than invertebrates and among vertebrates; cold-blooded species like turtles have longer repeat arrays (Chambers and MacAvoy, 2000). This is a very important factor and should be taken in consideration especially when trying to isolate microsatellites from such species since the results may be disappointing by producing fewer useful loci than expected.

The ability to amplify microsatellites by PCR provides a very convenient and efficient tool, whose results have high polymorphism capable of displaying many alleles per locus (Straughan, 2000, Comstock *et al.*, 2000). The procedure uses labelled primers and bands are visualised by autoradiography, fluorescency or silver staining. As for sequencing, the procedure of microsatellite analysis is becoming increasingly automated and hence enabling handling of many samples with variable microsatellite markers within a relatively shorter period (Fleischer, 1996; Strassman *et al.*, 1996).

Due to high level of variability at different loci, microsatellites have been widely used in genetic mapping and various population studies such as studies of genetic relatedness, individual identification and parentage as well as pedigree analysis

(Fleischer, 1996; Balding, 1999). Gene maps obtained from microsatellite analyses have become basis for qualitative trait locus (QTL) mapping. In different countries such as UK and USA individual identification using microsatellites (also known as DNA profiling), is a useful tool in routine laboratory forensic works (Balding, 1999). More studies have further shown that microsatellites isolated in one species can be successfully used in closely related species though results have been better with descendant than antecendent species (Morin *et al.*, 1994; Slate *et al.*, 1998; Gerloff *et al.*, 1999; Chambers and MacAvoy, 2000).

2.6.0 Studying genetic relatedness, parentage and pedigree

2.6.1 Human and domestic animal populations

In human and domestic animal populations, studies of relatedness and pedigree analysis have traditionally utilised various types of historical records. These records have been used in reconstruction of family trees as well as determination of other population parameters (Falconer and Mackay, 1997). However, this source of information is only useful where proper records are well kept and as such it cannot be utilised in case of partial records or where no information on breeding pattern of the population has been well documented. In domestic animals, breeding records have been useful in breeding programs that involve selection for particular favoured traits.

Records for pedigree and kinship are very important in breeding programs involving domestic animal species such as cattle, pigs, sheep, goats and poultry (Syrstad, 1988). Such information have been widely utilised in the improvement of

productivity of these animals through different methods such as selection and cross breeding. For example, selection and crossbreeding have been widely employed in programs that aimed at increasing milk production and beef for local breeds of cattle in the tropics (Syrstad, 1976, 1988; Zhang, 2000).

2.6.2 Wild animals

2.6.2.1 Observation and behaviour study

In free ranging populations of wild animals, studies of kinship and pedigree have frequently been done using long-term observational studies (Moss, 1988). Though this method can generate sufficient data to answer most of the population questions, it is limited with the time required to generate such data, ecological factors and resources. Furthermore, the method can be utilised efficiently for animals with short generation time like rodents and insects while it becomes of limited use for animals with extended generation time.

For large species of wild animals, long-term studies in the wild have been done on different species of animals such as primates, ungulates and others. In African elephants, such studies have been done by Moss (1988, 2001) in Amboseli National Park for more than 30 years, Foley (2002) in Tarangire National Park for over 8 years and Whitehouse and Hall-Martin, (2000) in Addo National Park for more than 6 years. Although the information obtained from these studies has been crucial for the understanding of the socio-biology of the African elephants, the methods used have been unable to resolve issues of genetic relatedness among individuals and paternity (Moss, 1988, 2001; Whitehouse and Hall-Martin, 2000). Thus, molecular

biology techniques through the use of biochemical and DNA markers are considered as the alternative methods to complement behavioural information in different natural populations of wild animals including elephants (Morin *et al.*, 1993, 1994; Gerloff *et al.*, 1999; Balding 1999; Beaumont and Bruford, 1999; Constable *et al.*, 2001; Whitehouse and Hall-Martin, 2000).

2.6.2.2 Use of genetic markers

Different studies have been conducted to establish genetic relatedness and paternity in wild animals in their natural environment. These studies have based on the use of different highly polymorphic genetic markers to resolve the genetic relatedness and parentage that could not be resolved by behavioural studies alone or where behavioural data are unavailable (Whitehouse and Hall-Martin, 2000). Initially, allozyme polymorphism was widely utilised in the study of polymorphism in natural populations (Queller *et al.*, 1993; Avise, 1994; Coltman *et al.*, 1999) before being replaced by DNA-based markers.

In African elephants, allozyme variation was utilised by Coetzee *et al.* (1993) to study polymorphism in free-living elephants in Kruger National Park. However, these markers had low lever of polymorphism, which limits their use, and hence alternatives markers were developed for studies involving different species of animals. Such markers include mtDNA that has been widely employed in various population studies (Kocher *et al.*, 1989; Balding, 1999). In their study, Kocher *et al.* (1989) used the combination of mtDNA markers and sequencing to determine polymorphism and relatedness in 110 species of different vertebrates and

invertebrates (birds, mammals, amphibians, reptiles, fish, insects and spiders). In other wild animals, mtDNA analysis together with sequencing or RFLP have been utilised in studies of different population parameters, species identification as well as in the attempt to determine genetic relatedness among closely related individuals and paternity analysis (Avise, 1994; Clarke *et al.*, 2001).

Different studies have made use of mtDNA to study both Asian and African elephant populations (Greenwood and Paabo, 1999; Nyakaana and Arctander, 1999; Ferdinand and Lande, 2000). Such studies demonstrated that in both Asian and African elephants, the high genetic relatedness among group/family members is due to the sharing of the same maternal ancestor (Nyakaana and Arctander, 1999; Ferdinando and Lande, (2000). It was also established from both behavioural and genetic analysis that Asian elephants may probably lack higher levels of social organisations such as bond groups and clan observed by Moss (1988) in African elephants. Apart from their wide use in population studies, mtDNA markers have shown low polymorphism and thus, they have been of limited use in kinship and parentage analysis.

Failures to resolve genetic relatedness among closely related individual has also been encountered in studies that utilised RAPD markers to study free-ranging wild animals (Ratnayeke *et al.*, 2002). However, the study was able to show that in racoons (*Procyon lotor*), phylopatry was biased towards females and individual females living in the neighbourhood were more related than others in the population.

The use of microsatellite markers is considered to be the best method for population studies, which include genetic relatedness and paternity analysis in wild species (Queller *et al.*, 1993). Microsatellites have been widely used in studies of different species of wild animals and as the marker of choice for paternity studies (Marshal *et al.*, 1998). According to the reviews by Beaumont and Bruford (1999) and Balding (1999), microsatellites analysis has been widely applied in conservation genetic studies of different animal species and birds such as Ethiopian wolf (*Canis simensis*), bears (*Ursus* spp) and greenbul (*Andropadus virenis*) as well as in human forensic studies.

Furthermore, microsatellites have been used for kinship and paternity analyses in savannah baboons, *Papio cynocephalus* (Altimann *et al.*, 1996), bonobos (*Pan paniscus*) (Gerloff *et al.*, 1999), chimpanzee, *Pan troglodytes schweinfurthii* (Morin *et al.*, 1993, 1994; Constable *et al.*, 2001), Soay sheep, *Ovis aries* (Coltman *et al.*, 1999) and red deer, *cervus elephas* (Marshall *et al.*, 1998). Although mating behaviour was able to predict paternity as determined by microsatellite analysis in primates, it however, failed to predict paternity in Soay sheep (Morin *et al.*, 1993, 1994; Altimann *et al.*, 1996; Coltman *et al.*, 1999; Constable *et al.*, 2001). A similar problem is likely to occur in elephants. This is because detailed records for different elephants populations are usually unavailable making behavioural data incomplete and less useful in complementing genetic data (Moss, 1988; Whitehouse and Hall-Martin, 2000).

The application of microsatellites for genetic relatedness and parentage in elephants has lagged behind other wild animals like primates and ruminants. This might be due to the fact that isolation of microsatellites in the elephants has only been done just recently (Nyakaana and Arctander, 1998; Eggert *et al.*, 2000; Comstock *et al.*, 2000) and probably due to the lack of microsatellites isolated from closely related species as is the case of primates that have indeed utilised human microsatellite markers (Morin *et al.*, 1993, 1994; Altimann *et al.*, 1996; Gerloff *et al.*, 1999; Constable *et al.*, 2001). Thus, few studies have utilised microsatellite markers in studying African elephants.

They include, the study of population genetic structure of African elephants in Uganda by Nyakaana and Arctander (1999) and the study of post-bottleneck genetic diversity among elephants in Addo and Kruger National Parks, South Africa by Whitehouse and Harley (2001). More studies have been recently done on the genetic diversity and phylogeography of both Savannah and forest elephants using both mtDNA and microsatellite markers (Eggert *et al.*, 2002; Nyakaana *et al.*, 2002). However, the currently available elephants specific microsatellite markers have shown very high polymorphism for both African and Asian elephants (Comstock *et al.*, 2000), such that they provide an enormous potential for studying elephant populations.

For other wild animals, microsatellites have been used in the study of genetic structure and paternity analysis of wild baboons, *Papio cynocephalus* (Constable *et al.*, 1995; Altmann *et al.*, 1996). Moreover, different studies have utilised

microsatellites to study population structure, species identification and phylogeography of brown bear, *Ursus actos* (Hoss *et al.*, 1992; Gerloff *et al.*, 1995; Woods *et al*, 1999; Clarke *et al.*, 2001). Similar studies have also been done in American black bear, *Ursus americanus* (Wasser *et al.*, 1997; Woods *et al.*, 1999), Malayan sun bear (Wasser *et al.*, 1997) and Polar bear, *Ursus maritimus* (Paetkaeu and Strobeck, 1995).

In addition, microsatellites have been also used in studies of population structure and phylogenetic studies of different animal species such as island fox, *Urocyon littoralis* (Goldstein *et al.*, 1999), horseshoe crab, *Limulus polyphemus* (Orti *et al.*, 1997) arctic fox, *Alopex logopus* and silver fox, *Vulpes vulpes* (Zoton and Filistowicz, 2001). Furthermore, studies have shown that Bovine microsatellite markers were highly conserved and could be useful in studying Red deer (*Cervus elephas*), Sika deer (*Cervus nippon*) and Soay sheep (*Ovis aries*) (Slate *et al.*, 1998).

Microsatellite have also been utilised in various studies such as amplification and genotyping of Alpine marmots, *Marmota marmota* using hair samples (Goossens *et al.*, 1998) and in molecular tracking of Mountain lions *Puma cancolor* (Ernest *et al.*, 2000) and wolf, *Canis lupus* (Lucchin *et al.*, 2002). Apart from terrestrial animals, microsatellites have also been useful in genetic studies involving kinship and individual identification of marine species such as whales (Valsecchi *et al.*, 1998), dolphins (Parsons, 2001) and fish (Tessier and Bernatchez, 1999).

The list of free-living marine and terrestrial animals in which microsatellite markers have been utilised for studying population genetics, phylogeography and evolutionary history highlighted above cannot be completed and is still expanding (Eggert *et al.*, 2002; Nyakaana *et al.*, 2002). However, it shows that the use of microsatellites in molecular and conservation genetics has a very big potential that should not be underestimated. Furthermore, the combined use of microsatellite analysis and non-invasive sampling techniques can invaluably be used in any study of kinship, pedigree and other genetic aspects of free-living organisms in their natural habitat (Taberlet *et al.*, 1999).

2.7.0 Collection of samples from wild game

2.7.1 Sampling methods

Several techniques have been widely used to collect samples from wild animals for different purposes such as disease diagnoses/investigations and research (see reviews by Taberlet *et al.*, 1996, 1999; Kohn and Whyne, 1997). These techniques can be broadly categorised into two major groups, which include invasive and non-invasive sampling methods. Invasive methods have been further sub-divided into destructive and non-destructive sampling based on the fate of the animal to be sampled (Taberlet *et al.*, 1999).

2.7.1.1 Invasive-Destructive and non-destructive sampling

Destructive sampling involves killing of the animal to obtain tissues for genetic and related studies. It was commonly used for isoenzyme studies and mitochondria DNA analysis before PCR was discovered. However, this method is being abandoned by

most researchers due to its consequences on the study animals and other logistics like obtaining permits to study endangered or rare species (Khon & Wayne, 1997; Taberlet *et al.*, 1999). On the other hand, non-destructive methods involve invasive techniques that enable the animal to be captured and taking samples such as tissue biopsy or blood. However, some non-destructive techniques allow collection of samples from the animal without immobilising or catching it such as the use of biopsy dart gun (Taberlet *et al.*, 1999).

2.7.1.2 Non-invasive methods.

The methods under this category have been defined by Taberlet *et al.* (1999) to include any method that will enable a required sample to be obtained after it has been left behind by the animal. The samples should be taken without catching the animal or causing any noticeable disturbance. The method is very useful and at times the only available means when obtaining samples from rare, endangered species or large mammals where immobilisation procedure may be contraindicated or very costly. It is also a method of choice when handling of the animals is likely to cause serious post-sampling consequences or where animals to be sampled require expensive handling techniques and/or permits which are not easily obtainable. Further to that, it can be used in conditions where logistics required to handle the animal and ensure its safety are unavailable (Hoss *et al.*, 1992; Constable *et al.*, 1995; Khon and Wayne, 1997). It is because of the above factors, non-invasive sampling is increasingly becoming the main method of obtaining samples from free ranging terrestrial and marine animals.

The most common samples collected non-invasively in free ranging wild animals include shed hair, faeces, urine, feathers, buccal cells from food wadges, sloughed skins (for snakes and whales), egg shells, skulls in owl pellets, left over of prayed species, tissues of naturally dead animals, fingernails and horns or tusks (Khon *et al.*, 1992; Constable *et al.*, 1995; Khon and Wayne, 1997; Taberlet *et al.*, 1996, 1999; Taberlet and Luikart, 1999).

These samples have been widely used in wildlife genetic studies. DNA from hairs of wild animals has been used to study alpine marmots (Goossens *et al.*, 1998), chimpanzee (Morin *et al.*, 1993, 1994; Constable *et al.*, 2001) and elephants (Greenwood and Paabo, 1999). Sloughed skin was used by Valsecchi *et al.* (1998) to study the population genetics of whales. DNA obtained from faeces has been used in studies of brown bear (*Ursus arctos*) (Khon *et al.*, 1992), Polar bear (*Ursus maritimus*) (Khon *et al.*, 1995) and wild savannah baboons, *Papio spp* (Constable *et al.*, 1995; Frantzen *et al.*, 1998).

Other studies using DNA from faeces involved the study of sheep and reindeer (Flagstard *et al.*, 1999), bonobos (*Pan paniscus*) (Gerloff *et al.*, 1995), Malayan sun and North American black beers (Wasser *et al.*, 1997), Mountain lions *Puma concolor* (Ernest *et al.*, 2000), African elephants (*Loxondonta africana*) (Nyakaana and Arctander, 1999) and dolphins (Parsons, 2001). Faeces have also been used in different studies that involve determination of the prey, predator or both from faeces (Farrel *et al.*, 2000; Symondson, 2002). In human, the use of DNA obtained from

intestinal cells shed in faeces was first developed for diagnosis of colorectal tumours (Sindransky *et al.*, 1992).

Molecular scatology has the potential of contributing immensely in wildlife genetic studied and if well exploited, it can provide information on the behavioural, socio-biology, census population size, home range and territory size, effective population size, genetic variation and phylogeography of the population (Khon and Wayne, 1997; Taberlet *et al.*, 1999). However, the use of DNA obtained from faeces for different studies of wildlife populations remained under-utilised in the early 1990s due to a number of limitations (Taberlet *et al.*, 1996). The major limitation has been the presence of low quantity and quality of host-specific DNA in faeces, which differ significantly among samples, presence of PCR inhibitors in faeces and presence of different types of DNA in faeces apart from that of the host (Taberlet *et al.*, 1996; Khon and Wayne, 1997).

In addition, there is high potential of cross contamination between samples during sample handling and analysis (Taberlet *et al.*, 1996, 1999; Kohn and Wayne, 1997). As a result, studied involving nuclear DNA obtained from faeces have encountered a number of drawbacks when using microsatellite markers as opposed to mtDNA markers. These include PCR high incidence of generated-false allele, sporadic contamination and allele dropout in a heterozygous genotype thus causing incorrect genotyping of an individual (Taberlet *et al.*, 1996).

The use of multiple tubes proposed by Taberlet *et al.*, (1996) has been shown to reduce these errors when using DNA derived from faeces although it has limitation on the number of samples which can be analysed as well as the costs of analysing large number of samples. Other alternatives techniques developed to enable full utilisation of scat DNA include the application of special faecal DNA extraction methods using DNA binding matrices such as magnetic beads (Flagstard *et al.*, 1999; Reed *et al.*, 1997) or silica (Wasser *et al.*, 1997). Commercially produced DNA extraction kits (such as QIAamp® stool kits, QiAgen Inc, CA, USA) and DNA cleaning kits (such as GeneClean® kits, QiAgen Inc, CA, USA) are currently available in the market. Thus, extraction and cleaning of faecal DNA using these kits has been shown to remove most the PCR inhibitors, originating from scat (Wasser, *et al.*, 1997).

2.7.2 Sample preservation and storage

The success of using faeces as a source of DNA depends on the preservation and storage methods so as to enable the samples to be taken from the field up to the laboratory for DNA extraction as in most cases these two places are far apart. And in some cases, faeces are not easily accessible due to different factors related to the animal or its surrounding environment. Studies to assess effectiveness of different preservation methods for the purpose of obtaining relatively high quality DNA have been done on different species such as bear and baboon faeces (Wasser *et al.*, 1997; Frantzen *et al.*, 1998; Murphy *et al.*, 2000). Wasser *et al.*, (1997) proposed the use of silica with storage at room temperature and 90% ethanol with storage at -4^0C or

-20^{0}C, whereas Murphy *et al.* (2000) assessed and proposed the storage of oven-dried samples at room temperature or -20^{0}C on bear faeces.

In a different study, Frantzen *et al.* (1998) tested and found that the use of DETs (DMSO/EDTA/Tris/salt) and storage at –20^{0}C, provided good quality DNA from baboon faeces with amplification rate of 64%. Another study by Murphy *et al.* (in press) tested five preservation methods of brown bear faeces at varying storage interval and found that 90% ethanol had the highest amplification success. However, Frantzen *et al.* (1998) established that amplification rate was very high for mtDNA and small fragments of nuclear DNA while it was low for medium and long fragments of nuclear DNA. This is because DNA in faeces degrades fast leaving many short and very few long fragments (Taberlet *et al.*, 1996). Because of the above limitations, amplification of scat DNA has been restricted to the use of mtDNA or short nDNA fragments suitable for microsatellite analysis (Frantzen *et al.*, 1998).

Thus, when all necessary measures are taken to avoid cross contamination between samples together with appropriate preservation and storage techniques, faeces can provide DNA with amplification rate higher than 75% and successful genotype scoring (Murphy *et al.*, in press). These advancements are likely to revolutionarise the use of molecular scatology, which has already showed enormous potentials in the studies of free-ranging animals in their natural habitat with minimal interference.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was done in Tarangire National Park (TNP), in Arusha region, Tanzania. TNP is located in the northern tourist circuit of Tanzania, which include the Serengeti, Lake Manyara, Arusha and Kilimanjaro National Parks as well as Ngorongoro Conservation Area Authority (NCAA). The Park covers an area of 2600 km^2 and lies between 3°40' - 5°35'S and 35°45' - 37°00' E. TNP receive an average annual rainfall of 665mm with variations occurring between different years. For example, TNP received very high rainfalls in 1979 and 1987 while it experienced severe drought in 1983 and 1993 (Foley, 2002). Monthly rainfalls show patterns that exhibit two distinct seasons, the dry season occurring between June and November and wet season between December and May (Foley, 2002).

TNP is dominated by two different types of soils, the dark alluvial soils in the riverine areas surrounded by low ridges of red loam soils of Precambrian rocks origin. These types of soils support a wide range of vegetation that fall within nine different vegetation subtypes or zones (Lamprey, 1963). The main vegetation type on alluvia soils is microphyll-wooden savannah, with *Acacia tortolis*, *Maerua* sp., and *Grewis* sp., intermixed together with Baobab (*Adansonia digitata*), sausage trees (*Kigeria africana*) and different perennial grasses. The ridges are covered with less deciduous woodland dominated by *Combretum zeyheri*, *Commifiphora* sp., and

Dalberghia melanoxylon, the African ebony. The southern part of the Park has large, seasonal swamps, which dry out during the dry season (Foley, 2002).

Tarangire River, traverses most parts of the park and the entire Tarangire ecosystem covering about 20,500 km^2. The river is the main source of water for the large number of migratory and resident animals especially during the dry season (Kidegesho *et al.*, 2000).

3.2 Study population

The elephant population in Tarangire has been studied since 1993 and the whole population has been sub-divided into three subpopulations (Foley, 2002). These include northern (NSP), central (CSP) and southern subpopulations (SSP) based on the specific parts of the park, which the animals use during the dry season (Moss, 1988). According to Foley (2002), the core area used by the NSP during the dry season include an area of about 695 km^2 of the northern part of the Park which extend to Lolkisale GCA during the wet season. The dry season home range of CSP is confined to 1090 km^2 north of Silale swamp and extends eastward to the southern parts of Lolkisale GCA during the wet season. However, the SSP use the southern part during the dry season and move outside the Park for the remainder of the year utilising the thick bushland as far as about 64 km south east of TNP. The home range of the three elephant subpopulations in Tarangire NP is shown in Figure 3.1.

The NSP is the most studied and all females and bulls have been identified using photographic files, ear markings, ear vein patterns and permanent features on the

body and these formed the main study group during the present studies. The NSP is made of 32 elephant families which belong to three clans, the North clan with 20 families, S-Camp and Jericho clans with 7 and 5 families, respectively (Foley, 2002). Based on the nature of the families with regard to the age structure of adult females and the composition of the families, all families in Northern subpopulation have been categorised as normal or altered families (Moss, 1988; Poole, 1989b; Foley, 2002). According to Foley (2002), normal families consist of one or more older females over 30 years of age as compared to altered families that have one or several adult females younger than 30 years.

Study animals included identified members of female families from NSP together with eight and six unidentified females from both the CSP and SSP, respectively. For the NSP, 9 families were sampled consisting of four and five families classified as altered and normal groups, respectively. The family groups sampled and the number of individuals per group is summarised in Table 3.1.

The number of individual animals sampled was different among families and for four families (A, I, L and X), efforts were made to obtain samples from all individuals in respective family. The rest of the families (C, D, T, Va, W), samples were obtained from only three adult females of over 17 years age. However, samples from some individual animals in A and L families could not be obtained due to logistical problems.

The choice of females over 17 years in some families of NSP, CSP and SSP (Table 3.1) was based on the assumption that all animals above 17 years should have survived the heavy poaching period either as adults or juveniles and hence remained in their intact families or joined with others to form altered families composed of unrelated individuals. This was aimed to assess the differences in genetic relatedness among adult females of that age category in altered and normal families as an indirect measure of poaching pressure on the family structure which will be done in future studies.

According to Dr Charles Foley of the Tarangire Elephant Project (TEP), (personal communication) there are about 40 elephant bulls over the age of 30, which dominate most of the mating. In addition, there are 88 bulls over the age of 25, which could potentially mate, though they are unlikely to get the chance. Thus, faecal samples were collected from 10 known breeding bulls over the age of 30 and two tissue samples from two male elephants that were found dead in the Park due to natural causes. The selection of the bulls ensured that all the dominant breeding bulls were sampled. The tissue samples were kindly provided by Dr. Charles Foley. Samples from males were aimed for use in paternity analysis.

Table 3.1: Families/groups of elephants sampled in TNP

Subpop	Group	Status	Group size	Number sampled	Number-offspring	Target
NSP	A	Normal	24	19	11	All
NSP	C	Altered	-	3	0	Adult females > 17 years old
NSP	D	Normal	-	3	1	Adult females > 17 years old
NSP	I	Altered	11	11	8	All
NSP	L	Normal	14	10	6	All
NSP	T	Normal	-	2	0	Adult females > 17 years old
NSP	Va	Normal	-	3	0	Adult females > 17 years old
NSP	W	Altered	-	3	0	Adult females > 17 years old
NSP	X	Altered	7	6	3	All
CSP	CSP		8	8	0	Adult females > 17 years old
SSP	SSP		8	6	0	Adult females > 17 years old
Males	Males		40	12	0	Males over 30 years old
Total		-	-	86	29	-

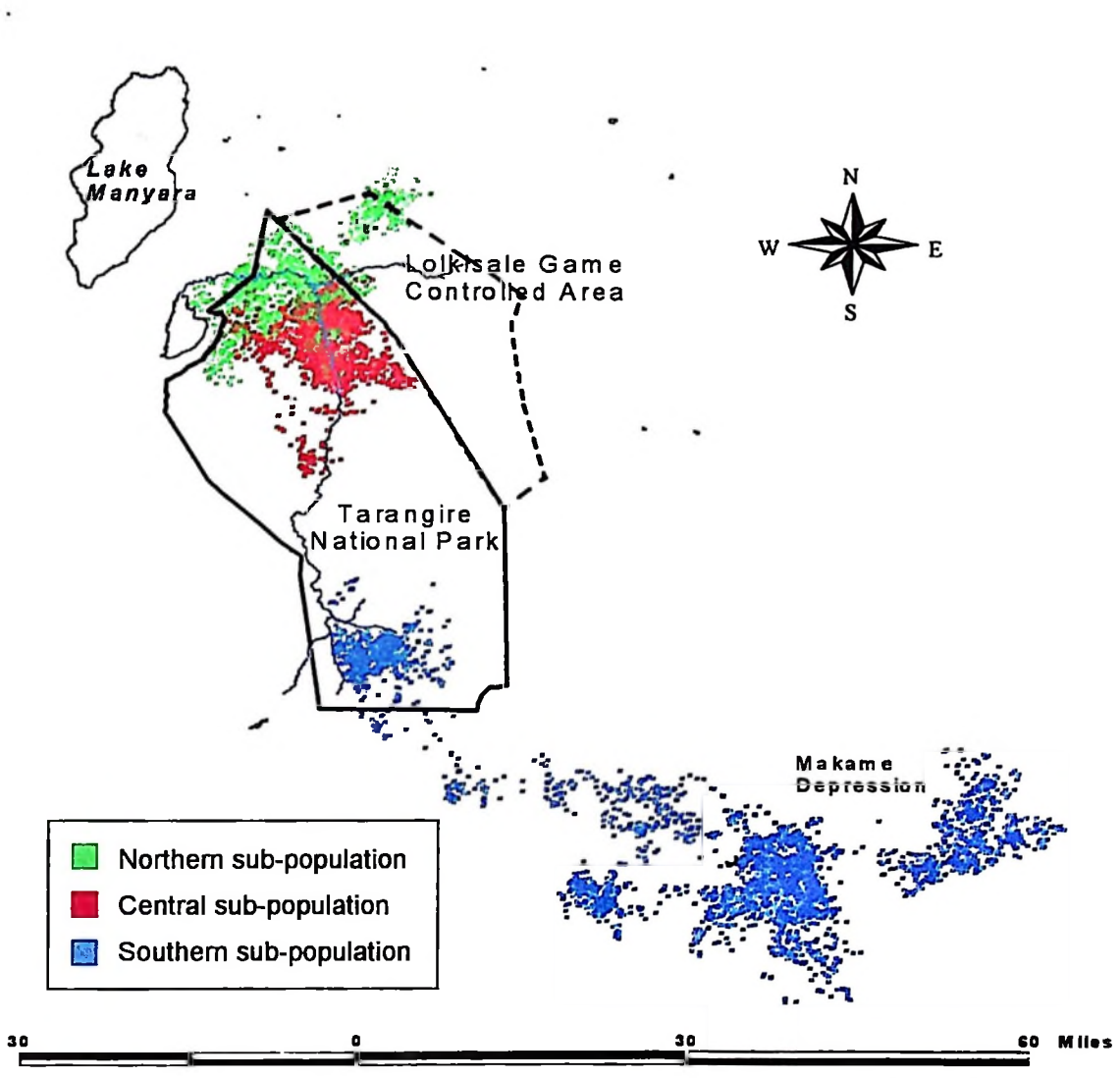

Figure 3.1: The map of Tarangire National Park showing the study area and the three subpopulations

3.3 Sample collection and preservation

Freshly voided faecal samples were collected from elephants after confirming the identity. Samples were collected from adult females and offspring from NSP as well as bulls. Animal identification was done using photofiles kindly provided by Dr. C. Foley, the Director of Tarangire Elephant Project (TEP). Faecal samples were picked with a gloved-hand by scratching the outer most part of the dung, which contains mucous and high concentration of cells from the intestinal mucosa. About 30g of faeces was mixed by gloved-hand, put in a 100 ml plastic vials and then absolute ethanol (AnalaR, 3:1 sample: ethanol v/v) or silica (Sigma) (4:1 silica: sample v/v) was added as a preservative. Samples obtained from eight bulls were preserved in silica. Ethanol and silica have been shown to be good preservatives that maximises the chances of getting high quality DNA from scat (Wasser *et al.*, 1997; Frantzen *et al.*, 1998; Murphy *et al.*, (in press).

For elephants from the CSP and SSP, samples were collected from adult females aged over 17 years in different families belonging to respective subpopulation. Sample collection was done in two consecutive days and at a distance far enough to avoid sampling from the same or related families. In the field, samples were stored at room temperature while in the laboratory at SUA samples were stored at –20°C before DNA extraction.

3.4 DNA extraction

Samples were first air-dried at room temperature overnight to remove ethanol and then freeze-dried using Wagtech freeze-drying machine (EC Apparatus, NY, USA).

The dried samples were mixed by a gloved-hand without crushing the grass. This allowed separation of undigested grass materials and the powdered portion of the faecal material was sifted out using a 2.0 mm^2 wire sieve. DNA was extracted from all 86 samples using QIAamp DNA mini stool Kit (QiAgen Inc., Germany, Cat. No. 51309) with the procedure and reagents supplied within the kit. Essentially, 200 mg of faecal powder per sample was transferred in duplicate into 10 ml centrifuge tubes, then 1.6 ml of QiAgen ASL buffer was added in each tube, vortex-mixed for 1 min to soak the sample and thereafter incubated at 70°C for 1 h to lyse the faeces.

After incubation, the tubes were centrifuged at 13,000 rpm for 3 min and the respective supernatants were carefully pipetted out and transferred into new 1.5 ml tubes. Spinning at 13,000 rpm for 3 min was done for the second time and the supernatant was transferred into 2 ml tubes containing InhibitEx tablets, which are designated to remove PCR inhibitors present in the dung. The mixture was incubated at room temperature for 1 min to allow InhibitEx to bind the inhibitors. Thereafter, the contents were spun at 13,000 rpm for 3 min. About 500 µl of the supernatant was obtained and transferred into new 1.7 ml microcentrifuge tubes containing 25 µl (20mg/ml) of Proteinase K (Sigma, WI, USA). This was followed by addition of 45 µl (20 mg/ml) of RNAse A (Sigma, WI, USA).

The mixture was vortexed and then incubated at room temperature for 2 min, followed by addition of 500 µl of QiAgen AL buffer, vortex-mixed and incubated at 70 °C for 1 h. After incubation, 500 µl of absolute ethanol (AnalaR) was added to each tube and then 650 µl of the lysate was transferred onto respective QIAamp spin

columns containing DNA-binding silica membrane. After loading of the samples, the spin columns were centrifuged at 13,000 rpm for 30 sec. Thereafter, the columns were washed with 500 µl of AW1 and of AW2 with spinning at 13,000 rpm for 30 sec and 1 min for each QiAgen buffer, respectively. The bound DNA was eluted from the columns by addition of 200 µl of QiAgen AE elution buffer (pre-incubated at 70°C) and the mixture was incubated at room temperature for one minute. The final column spin at 13,000 rpm for 1 min was done to obtain all the DNA from the silica membrane. The eluted DNA was collected in 1.7 ml microcentrifuge tubes and stored at –20°C. The extracted DNA was freeze-dried. All the DNA extractions were performed at SUA.

3.5 Gene Clean

Since DNA from scat contains many impurities, some of which are PCR inhibitors, a cleaning stage is recommended to reduce the inhibitors and concentrate the elephant specific DNA which is in very low concentration in faeces (Taberlet *et al.*, 1996; Wasser *et al.*, 1997). This work was done at the Centre for Conservation Biology, Department of Zoology, University of Washington (USA). Freeze-dried DNA samples were reconstituted by adding 200 µl of deionised water and thereafter cleaned using Gene clean Kit (QiAgen, CA, USA) using the protocol provided by the manufacturer. Essentially, 600 µl of NaI/ TBE modifier buffer was added to 200 µl of the DNA sample (3 NaI/TBE: 1 DNA v/v) in a 1.7 ml microcentrifuge tube, followed by addition of 10 µl of well mixed EZ Glassmilk® (aqueous suspension of silica matrix)

The mixture was incubated at 55°C for 12 min to allow silica to bind DNA. The tubes were inverted every 3 minutes during incubation to resuspend and optimise the DNA binding on silica matrix. After incubation, the tubes were centrifuged at 13,000 rpm for 20 sec to pellet the Glassmilk® and the supernatant was discarded. The pellet was washed three times with 200 µl of QiAgen New Wash solution. Between each wash, the tubes were centrifuged at 13,000 rpm for 20 sec to pellet the Glassmilk®.

After the final wash, the New Wash was pipetted off and the tubes were heated with lids open at 55°C for 10 min to remove ethanol. Finally, eluting of DNA was done by adding 50 µl of QiAgen Elution Solution to the pellet, re-suspending by vortexing followed by spinning at 13,000 rpm for 2 min to pellet the Glassmilk®. The supernatant was immediately transferred to new tubes by pipetting carefully to avoid drawing Glassmilk®. The DNA obtained was stored at –20°C prior to PCR amplification.

3.6 DNA amplification

PCR amplification was carried out using between 10-100ng of faecal DNA and elephant-specific microsatellite markers previously described by Nyakaana and Arctander (1998) and Comstock *et al.* (2000) (Table 3.2). During the initial PCR runs, 24 randomly selected DNA samples were amplified for all 16 microsatellite markers to assess their amplification status, level of polymorphism and the ability of the marker to generate allele data, which could be scored on the ABI to obtain individual genotype. Only 8 highly polymorphic microsatellite markers were

subsequently selected and used for amplification of the remaining samples (Table 3.2). Thus, the data presented in this dissertation is based on the PCR amplification using 8 selected microsatellite markers. A total of 86 elephants were genotyped which included 29 offspring and 31 adult females from NSP, 8 adult females from the CSP, 6 adult females from the SSP and 12 bulls over 30 years of age.

A 25 µl volume PCR was done using multiwell polypropylene microplates (MJ Research Inc., MA) and performed on GeneAmp® System 9700 (Applied Biosystem- ABI, USA) thermocycler. The PCR profile involved initial heating at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 2 min. The 25 µl PCR mixture contained 62.5 mM $MgCl_2$, 5 µg of BSA, 0.5 mM of each dNTP, 10 µM of each primer, 2.5 µl of 10X PCR buffer (Promega, WI, USA) and 1 µl of scat DNA template. The amplification utilised TaqStart PCR, which is a modification of hot start PCR (Kellogg *et al.*, 1994) using 0.5 U Taq DNA polymerase (Promega, WI, USA) mixed with 0.7 µM TaqStart™ Antibody (CLONTECH, CA, USA) and the antibody dilution buffer (1:1:4 v/v).

Table 3.2: Primer sequences for both forward and reverse primers as well as annealing temperature for the 16 elephant specific microsatellite markers tested with 24 samples

Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Ta# °C	Size (bp)
FH129	TGA TTT GAT GTC CCT TCC AG	TTG GCT AAA ATG CCT ATC ACT C	55	155-175
FH48*	GAG TCT CCA TAA TCA AGA GCG	CCT CCC TGG AAT CTG TAC AG	55	160-180
FH60	CAA GAA GCT TTG GGA TTG GG	CCT GCA GCT CAG AAC ACC TG	55	145-165
FH71	GGG ATT GGC TAA AAT AG	CTA AGC ACA TCA GGG AC	55	70-110
FH40	GGC TTT CTA GCC ACC TCC TTC	GCT CAC ATT CAC TTG CTG ACC	55	230-260
FH94*	TTC CTC CCA CAG AGC AGC	ATT GGT TAA TTT GCC AGT CC	55	225-240
FH39	GTA TTC CTG GGC ATT CCA TG	CTT GGA ATA TGA CCC TGT TTG	55	235-255
FH103*	TGT GCT GCC ACT TCC TAC AC	GAT GTT GAG ACA GTT CTG TAA G	55	145-155
FH102*	CTT CAT TAC TGA CCT AAA CGA G	GGA CAG GGC TGG AGA AAT ATG	55	160-200
FH67*	GCT TCT CTA GAA ATG TGT ATG C	GGC GTA TAG GAT AGT TCC AC	55	90-105
FH19*	GAA GCT CAT GGT CAA GGT CAC	CTG CAT ACT CAT CGA AG TCA CC	55	185-215
FH127	ACT GAC CGG GAA GAG GAA GT	AGG TTT CTG AGC TGG ATT GG	55	160-280
FH153*	CAT GGG CCT AAG CTA AAA CG	GTC ACA TGG GGT TGC TAC	55	150-300
FH126	TCT GAT AGG CTG GTG TAA GCT G	TCT CTC CTC CCT TCC CTC TC	55	90-120
MS03	CAT ATG AAC ATA CCG GAA C	GAA ACT CCT CGA GTA GTA GAA	55	145-150
MS04	GGG ACA GTG TGC ATA A	TTA TGT CTG CAT AGA CAG GTT GG	55	150-160

Source: Nyakaana & Arctander (1998) for MS03 and MS04 and Comstock *et al.* (2000) for the rest of the primers. All forward primers were labelled with FAM except for FH67 and FH94, which were labelled with VIC (Applied Biosystems, Inc., USA). Primers marked with star (*) were selected for subsequent PCR amplifications. Ta#: Refers to annealing temperature.

3.7 Gel Electrophoresis

PCR products were electrophoresed at 80V for 80 min in 1.5% agarose gel prepared and run in 1X TBE (Tris-Boric acid–EDTA) buffer. A 7 µl loading mixture was made of 6.3 µl of DNA and 0.7 µl of 10X loading dye and only 6 µl of the mixture was loaded in each well. A ladder solution was also prepared using 0.25 µl /lane (50µg/ml) of 100 bp DNA ladder (Promega, WI, USA), 0.6 µl of 10X loading dye and 5.15 µl of deionised water. As for PCR products, 6 µl of the 100 bp ladder solution was loaded in the first and the last wells, respectively.

After electrophoresis, the gel was stained using a solution containing SYBR® Gold nucleic acid gel stain (Molecular Probes, OR, USA) in 1X TBE buffer (1:10 000 v/v) for 15 min and the gel picture was taken using a computer-connected automatic camera. The gel images were analysed using Bio Rad Q101 programme (ABI, Applied Biosystem, USA) and the resulting DNA band intensity was used to determine the quality and quantity of the DNA by comparing with the bands of the 100bp ladder. This facilitated in the estimation of the dilution factor before loading the samples onto the ABI Prism® 3100 sequence Analyser (Applied Biosystems-ABI, USA) for fragment size determination.

3.8 DNA microsatellites analysis

The PCR products for the initial 16 and thereafter 8 microsatellite markers were subjected to DNA fragments analysis using the ABI Prism® 3100 sequence Analyser (Applied Biosystems-ABI, USA). This involved loading of 1.0 µl of PCR products either after dilution or without as shown by their band intensity in comparison with

the 100 bp ladder such that approximately 10 ng of DNA was used. The final mixture loaded onto the ABI Prism® 3100 sequencer analyser was made up of 1.0 µl PCR products in 10 ul of formamide solution. Running of the ABI Prism® 3100 sequencer analyser was done according to manufacturer's guide and the data obtained was directly stored into the computer. Viewing of the fragments, analysis and scoring of the microsatellite data was done using ABI Prism® GeneScan® analysis software, version 3.7 for Windows NT® platform.

3.9 Data analysis

3.9.1 Population statistics

The data obtained was subjected to statistic analysis in order to determine the genetic polymorphism in the sampled population. This was done through analysis of genetic diversity and allele frequencies for the 8 microsatellite markers using CERVUS 2.0 (Marshall *et al.*, 1998; Slate *et al.*, 2000). Genetic diversity was determined by the total number of alleles in the population, average number of alleles per locus and heterozygosity values (both observed and expected heterozygosity).

3.9.2 Paternity analysis

Paternity assignment was done for 29 sampled offspring whose mothers were known and thus serving as known parent during paternity analysis. A total of 12 out of 40 breeding bulls sampled were used for parentage analysis making 30.0 % of the total potential fathers in TNP. The number of potential breeding bulls in TNP was determined based on the total number of males with the age of over 30 years old. This has been observed to be minimum age at which male elephants in TNP can

perform successful mating and are therefore more likely to sire more infants than males of ages below 30 years (Foley, 2002). The likelihood-based paternity analysis was instituted by CERVUS software 2.0 (Marshall *et al.*, 1998). In contrast to parentage analysis based on the process of exclusion by genotype matching between the parent and the offspring, likelihood was used to statistically distinguish non-excluded candidate parents due to mismatches with the offspring.

Paternity assignment was done by calculating the likelihood ratio for each candidate parent (i.e. the likelihood of parentage of that candidate parent relative to the likelihood of parentage of an arbitrary unrelated candidate parent), and by comparing the likelihood ratios of different candidate parents. Then, the LOD score (the natural log of the product of the likelihood ratios at each locus) was calculated for each candidate bull based on its genotype, the genotypes of offspring as well as known mother according to Marshall *et al.* (1998) and Slate *et al.* (2000).

A positive LOD score implies that the candidate parent is more likely to be the true parent than an arbitrary randomly chosen individual. In absence of typing errors, the true parent should have a positive LOD score and the most likely candidate parent is the candidate parent with the highest (most positive) LOD score. On the other hand, a negative LOD score implies that the candidate parent is less likely to be the true parent than an arbitrary randomly chosen individual. A negative LOD scores can occur when the candidate parent and offspring share common alleles at every locus (Marshall *et al.*, 1998).

If likelihoods are calculated without taking account of errors, any mismatch leads to a likelihood ratio of zero, meaning that the LOD score is undefined. A LOD score of zero implies that the candidate parent is equally likely to be the true parent as an arbitrary randomly chosen individual. In order to discriminate between non-excluded males, CERVUS used a statistic Delta to assess the reliability of assigning paternity to the most likely bull (Marshall *et al.*, 1998). Delta was obtained as the difference in LOD scores between the most likely bull and the next most likely bull among the bulls genotyped and if LOD of the next likely bull was very negative, the delta becomes large regardless the LOD value of the most likely bull (Marshall *et al.*, 1998).

The significance of delta values was assessed using simulation analysis done by CERVUS with predetermined parameters including number of males sampled, proportion of candidate males sampled, proportion of loci typed, rate of typing error, number of tests and confidence levels (Marshall *et al.*, 1998). Paternity assignment was therefore done using 30% as proportion of males sampled at both 95% (strict) and 80% (relaxed) confidence levels. Paternity analysis was also done by making an assumption that the 12 dominant bulls sampled did majority of the mating in TNP and hence represented nearly 100% of the effective population size (N*e*). Paternity assignments at both 30% and 100% simulations were compared.

Furthermore, individual exclusion probability was calculated for all 86 elephants sampled from TNP as the average probability of excluding a single unrelated candidate parent from parentage of a given offspring at one or more loci, assuming

no typing errors occur. The probability of excluding a randomly chosen candidate parent from parentage of an arbitrary offspring, given only the genotype of the offspring (P_E 1) and when given the genotypes of both the offspring and known parent of the opposite sex (P_E 2) were calculated from the genotype data. If one parent was known, the exclusion probability was calculated taking account alleles that are unambiguously descended from the known parent. The formulae for all of the above calculations are given in Marshall *et al.* (1998) and Slate *et al.* (2000).

CHAPTER FOUR

4.0 RESULTS

4.1 Genetic diversity of elephants in TNP

A total of 86 individual elephants from Tarangire National Park (TNP) were genotyped at eight microsatellite markers namely FH102, FH103, FH126, FH153, FH19, FH48, FH67 and FH94. The amplification and genotyping success was determined as the number of individual elephants producing a genotype at any given locus. For all microsatellite markers used in this study, FH67 and FH94 had the highest amplification and genotyping success per locus (91.9%) while FH48 had the lowest (81.4%) and the mean amplification and genotyping success was 87.8% (Table 4.1). However, four samples including two preserved in silica failed to amplify at all eight microsatellite markers. The genotypes of all 86 elephants sampled in TNP and the samples that failed to amplify at any of the 8 microsatellite markers are presented in Appendix 1.

Microsatelite allelic variation at all eight markers showed that all markers were highly polymorphic with a total of 60 alleles detected in the sampled population. The mean number of alleles per locus was 7.50 ± 1.035 (s.e.) and the most variable loci were FH153 and FH67 with 11 alleles each while FH94 was the least variable locus with 3 alleles (Table 4.1). Allele frequency was also calculated using CERVUS for all eight microsatellite markers in the entire population sampled and the results are shown in Figure 4.1. The alleles with the highest frequencies for each locus were 168bp (FH48), 193bp (FH19), 106bp (FH126), 167bp (FH153), 95bp (FH67), 226bp

(FH94), 177bp (FH102) and 151bp (FH103). The rare alleles in the sampled population included 156bp (FH48), 195bp (FH19), 109bp (FH126), 167bp (FH153), 95bp (FH67), 226bp (FH94), 177bp (FH102) and 151bp (FH103). The frequencies of the most common alleles for each of the eight loci ranged from 29.1% to 63.0% while the least frequent alleles for each locus had frequencies ranging from 0.6% (FH67, FH102 and FH103) to 16.9% (FH94).

The mean observed heterozygosity (H_O) for all eight markers was 0.701 ± 0.038 (s.e.) and varied from 0.519 (FH94) to 0.809 (FH19) while unbiased expected heterozygosity (H_E) per locus under Hardy-Weinberg equilibrium ranged from 0.538 (FH94) to 0.820 (FH126). The mean expected heterozygosity for all eight markers was 0.706 ± 0.037 (s.e.) (Table 4.1).

Table 4.1: Summary of amplification/genotyping success (GS) and heterozygosity values for 86 individual elephants from TNP.

Marker	k	N	GS%	H_O	H_E
FH102	5	78	90.7	0.603	0.601
FH103	6	77	89.5	0.701	0.666
FH126	10	75	87.2	0.773	0.820
FH153	11	76	88.4	0.789	0.781
FH19	7	72	83.7	0.806	0.771
FH48	7	70	81.4	0.629	0.664
FH67	11	79	91.9	0.785	0.809
FH94	3	77	89.5	0.519	0.538
Mean ± s.e	7.50 ± 1.035	76	87.8	0.701 ± 0.038	0.706 ± 0.037

Total number of alleles (k) = 60.

k = Number of alleles per locus, N= Number of individuals genotyped at each locus, GS = Genotyping success, H_O = Observed heterozygosity, H_E = Expected heterozygosity and s.e. is the standard error of the mean.

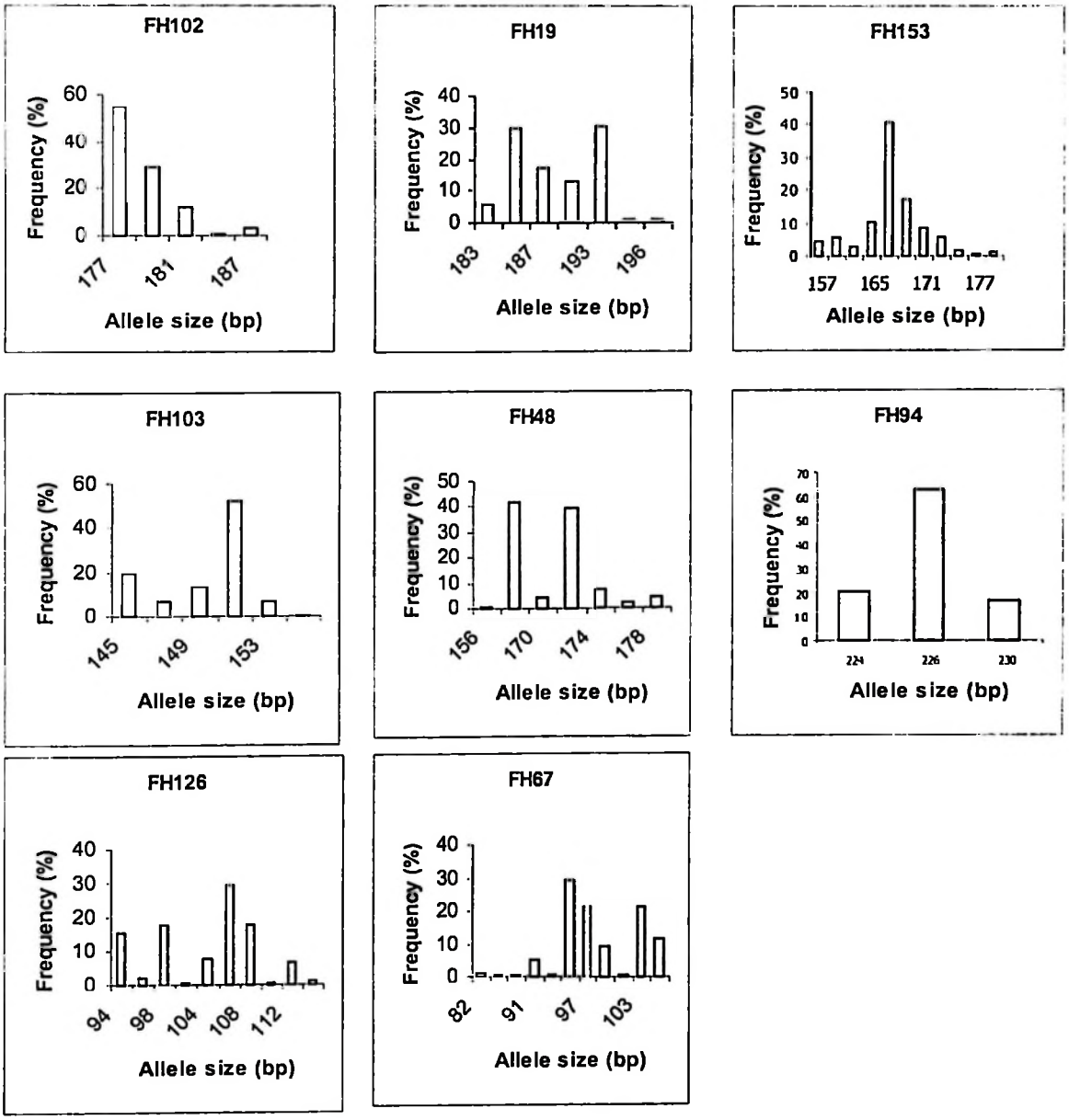

Figure 4.1: Graphical representation of allele frequency by locus for all 86 elephants sampled in TNP. The locus represented is indicated at the top of each graph.

The genotypes of all individuals sampled from TNP were subjected to analysis of the polymorphic information content (PIC), test for Hardy-Weinberg equilibrium (HWE) and null alleles frequency using CERVUS. PIC is a measure of informativeness of each locus and it is related to expected heterozygosity since both values assume HWE. On the other hand null allele frequency estimation is critical for the microsatellite markers to be used for parentage analysis since any locus to be used for such analysis must have null allele frequency of less than 5% (Marshall *et al.*, 1998)

The results for all eight markers depicted that the most polymorphic markers (FH153, FH126, FH19 and FH67) had the highest polymorphic information content (PIC) while PIC was low for FH94 and FH102 markers. The mean PIC was 0.66 ± 0.042 (s.e.) and the highest PIC was 0.790 for FH126 while FH94 had the lowest PIC (0.477). The PIC values for all loci showed that loci with the highest PIC had the highest expected heterozygosity values and vice versa (Table 4.2).

The results for both P_E (1) and P_E (2) were higher for loci with high number of alleles than those with low number of alleles corresponding to the informativeness of the locus as shown by PIC and heterozygosity values. P_E (1) ranged from 0.143 at FH94 to 0.461 (FH126) while the values of P_E (2) ranged from 0.286 (FH94) to 0.636 at FH126 (Table 4.2).

The test for HWE for all eight microsatellite markers revealed no significant departure from HWE expectation as summarised in Table 4.2. The results for null

frequency analysis were positive at four microsatellite markers (FH126, FH48, FH67 and FH94) and negative at the rest of the markers. Null allele frequency was the highest at FH48 (0.024) while FH103 had the lowest frequency of null alleles (–0.040). These results show that all microsatellite markers used in this study had the frequency of null alleles of less than 5% since the highest was 2.4% for FH48 and all markers were therefore suitable for paternity analyses.

Table 4.2: Polymorphic information content, HWE test and null allele frequency analysis for the eight-microsatellite markers.

Marker	PIC	P_E (1)	P_E (2)	HWE	NF
FH102	0.534	0.188	0.336	NS	-0.007
FH103	0.622	0.257	0.435	NS	-0.040
FH126	0.790	0.461	0.636	NS	+0.023
FH153	0.754	0.417	0.599	NS	-0.007
FH19	0.729	0.370	0.549	NS	-0.031
FH48	0.598	0.244	0.403	NS	+0.024
FH67	0.777	0.441	0.617	NS	+0.010
FH94	0.477	0.143	0.286	NS	+0.004
Mean ± s.e.	0.660±0.042	0.315±0.043	0.483±0.048	-	-0.003±0.008

PIC = Polymorphic information content, P_E (1) =Average exclusion probability (1), P_E(2) =Average exclusion probability (2); HWE = Hardy-Weinberg equilibrium test and NF= Null allele frequency estimate. NS = "not significant".

4.2.0 Paternity assignment for Tarangire elephants

Paternity assignment was done for 29 sampled infants with known mothers and 12 out of 40 potential breeding bulls in TNP. All individual elephants used in paternity analysis were genotyped at a minimum of four loci. Samples from two bulls (T130 and T148) failed to amplify at all loci and thus were excluded from paternity inferences leaving a total of 10 bulls to which paternity assignment was done.

In this study, paternity analysis was done for 30% and 100% as proportion of candidate males sampled in TNP and paternity tests were reported as resolved paternity at 95% (strict) and 80% (relaxed) confidence (Table 4.3). Assignment was also reported as unresolved by assigning one of the bulls as the most likely father due to very low LOD and delta values. Furthermore, five offspring were assigned to the candidate parents that were not the most likely parent and the tests were also reported as unresolved paternity cases because of the negative LOD values and delta values of zero (Table 4.4 and 4.7).

Paternity inference using 30% as the proportion of sampled males at 80% confidence enabled to assign 14 (48%) offspring to their potential fathers, leaving paternity cases of 15 (52%) infants unresolved (Table 4.3). Four out of the 14 offspring assigned to their potential fathers at 80% confidence were re-assigned to the same fathers at 95% confidence. The delta values used for paternity assignments at 80% confidence ranged from 1.374 to 2.483 while paternity assignments resolved at 95% confidence had delta values ranging from 3.869 to 6.966. The minimum delta value for paternity

tests resolved with 30% as proportion of males sampled were 3.32 and 1.58 for 95% and 80% confidence levels respectively (Tables 4.3 and 4.4).

The highest delta value of 6.699 obtained during paternity analysis means that the assigned candidate male was more than 800 times more likely to be the father of the offspring, whereas a delta value of 1.0 means that the assigned male was 2.72 times more likely to be the father of the offspring compared to the next more likely candidate male. The delta value of zero implies that the assigned male is as equally likely to be the father of the offspring as any other bull in the population.

Paternity assignment was re-analysed using the 12 bulls (as N_c) representing 100% of the proportion of males sampled, and paternity tests resolved were higher than that obtained with 30% (Table 4.3 and 4.4). The number of paternity cases resolved was the same at both 80 and 95% confidence and 24 offspring (83% success) were assigned to their potential fathers leaving paternity tests of only 5 (17%) infants unresolved (Tables 4.3 and 4.4). For 100% simulation, the delta values utilised for paternity assignment ranged from 0.273 to 6.699 and the minimum delta value was zero for both 80% and 95% confidence levels. With both assignments for 30% and 100% as the proportion of candidate males sampled in TNP, bulls assigned as potential father of each offspring were the same even when the paternity was unresolved though different LOD and delta values were used (Table 4.4). The details of both paternity analyses at both 30% and 100% (proportion of males sampled in TNP) are summarised in Appendices 2 and 3.

Table 4.3: Paternity assignment for 29 sampled infants in TNP with known mothers using 30% and 100% as proportion of males sampled

Level	Minimum delta	Confidence (%)	Tests	Success%
30% simulation (12/40)				
Strict	3.32	95	4(7)	14(25)
Relaxed	1.58	80	14(15)	48(52)
Unresolved	-	-	15(14)	52(48)
100% simulation				
Strict	0.00	95	24(29)	83(100)
Relaxed	0.00	80	24(29)	83(100)
Unresolved			5(0)	17(0)

Numbers in bracket show expected values of resolved paternity as predicted by simulation and tests refer to resolved paternity cases.

Table 4.4. List of males assigned to offspring with known mothers using 30% and 100% as proportion of males sampled in TNP

Known mother's name	Offspring-name	30% simulation				100% simulation			
		LOD	Delta	Confidence	Bull assigned	LOD	Delta	Confidence	Bull assigned
Adina	Adina '96 (F)	1.380	1.380	80%		1.380	1.380	95%	
Isabel	Isabel '97 (F)	2.489	1.764	80%		2.489	1.764	95%	
Iselle	Iselle '96 (M)	3.869	3.869	95%		3.869	3.869	95%	
Iselle	Iselle '99 (M)	0.346	0.346	ML		0.346	0.346	95%	
Lynne	Lynne '99 (M)	1.119	1.119	ML		1.119	1.119	95%	
Lorraine	Lauren	-0.562	0.000	NR	Aragorn	-0.562	0.000	NR	Aragorn
Lorraine	Lakini	2.529	1.700	80%		2.529	1.700	95%	
Leda	Lea	2.483	2.483	80%		2.483	2.483	95%	
Fng	Desdemona	1.205	1.205	ML		1.205	1.205	95%	
Ines	Inesita	-0.916	0.000	NR	TT3	-0.916	0.000	NR	TT3
Alexis	Alexis (F) 96	-1.127	0.000	NR		-1.127	0.000	NR	
Adria	Adria (F) '00	0.594	0.290	ML		0.594	0.290	95%	
Ines	Ines '99 (F)	3.351	2.150	80%	UNO	3.351	2.150	95%	UNO
Alison	Alison (F) '98	0.247	0.247	ML		0.247	0.247	95%	
Addo	Adina	2.151	2.151	80%		2.151	2.151	95%	
Ines	Rina	5.363	4.912	95%		5.363	4.912	95%	
Lynne	Lynne '96 (M)	2.210	2.210	80%	Achilles	2.210	2.210	95%	Achilles
Acacia	Allegra	2.118	0.959	ML		2.118	0.959	95%	
Adina	Adina (F) '98	-2.161	0.000	NR	Bombadil	-2.161	0.000	NR	Bombadil
Lorraine	Lynne	1.387	1.357	80%		1.387	1.357	95%	
Addo	Andy	4.988	4.988	95%		4.988	4.988	95%	
Adina	Ashley	6.966	6.966	95%		6.966	6.966	95%	
Isabel	Isabel '00 (F)	1.547	0.526	ML	Bruce	1.547	0.526	95%	Bruce
Laurens	Laurens 00 (M)	1.811	1.374	80%		1.811	1.374	95%	
Leda	Leda '99 (F)	2.589	1.582	80%		2.589	1.582	95%	
Amy	Amy (F) 99	2.396	0.642	ML		2.396	0.642	95%	
Isabel	Ivan	0.273	0.273	ML	Plato	0.273	0.273	95%	Plato
X-tusk	X-ley	1.497	1.101	ML		1.497	1.101	95%	
Addo	Addo (F) 00	-0.489	0.000	NR	Simon	-0.489	0.000	NR	Simon

ML= Most likely candidate parent, NR= Unresolved paternity assignment

4.2.1 Genotypic mismatches and error rate analysis

Table 4.5 shows genotypic mismatches between offspring and their mothers per locus on all 8 markers used in this study. There were 10 mismatches observed between the genotypes of mothers and their offspring. Among the loci used, FH67 had the highest number of mismatches (4), FH103 had three mismatches, FH19, FH153 and FH126 had one mismatch each while three loci (FH102, FH48 and FH94) had no mismatch. Between infants and their respective mothers, T160 infant and her mother (T156) were the only pair with the highest genotypic mismatches (three) at FH67, FH153 and FH103 loci. Other seven infants (T195, T155, T256, T200, T182, T161, T162) and their mothers had only one genotypic mismatch each

Based on the results of genotypic mismatches between the genotypes of offspring and their mothers shown in Table 4.6 and the number of genotypes compared between the offspring and their mothers at each locus, two loci (FH103 and FH67) had the highest error rates. The highest error rate was 0.224 for FH103 (with three mismatches) because only the genotypes of 26 out of 29 sampled infants were compared with their mothers' while FH67 (with four mismatches) had lower error rate (0.162). For all eight microsatellite markers, the error rate per locus ranged from zero to 0.224 and the mean observed error rate across all loci was 0.067. The results for null alleles detection revealed that only one locus (FH67) had a null allele.

Table 4.5: Genotypic mismatches between offspring and their mothers per locus

Locus name	Offspring ID	Offspring genotype*	Mother's ID	Mother's genotype*
FH67	T195	105, 105	T234	97, 97
FH67	T160	95, 97	T156	91, 105
FH67	T155	105, 105	T219	91, 97
FH67	T256	82, 82	T164	83, 85
FH19	T200	193, 193	T233	185, 187
FH153	T160	161, 167	T156	165, 173
FH126	T182	96, 108	T180	94, 98
FH103	T160	149, 153	T156	145, 147
FH103	T161	145, 159	T160	149, 153
FH103	T162	151, 151	T160	149, 153

*Genotypes for both offspring and mothers are shown by allele size in bp.

Table 4.6: Estimated Error rate analysis for the eight microsatellite markers

Locus name	N compared	N mismatching	Null allele(s)	Detection prob.	Error rate
FH102	25	0	0	0.188	0.00
FH103	26	3	0	0.257	0.224
FH126	29	1	0	0.461	0.037
FH153	28	1	0	0.417	0.043
FH19	20	1	0	0.370	0.068
FH48	19	0	0	0.244	0.000
FH67	28	4	1	0.441	0.162
FH94	29	0	0	0.143	0.000

N = number of offspring whose genotypes were compared with mothers' genotypes at each locus and detection prob. is the average probability of detecting a mismatch.

4.2.2 Mating success

Mating success of individual male elephants was assessed based on the number of infants assigned to each male during paternity analysis. For the paternity resolved using 30% as a proportion of sampled males in TNP, three bulls (7.5% of the potential breeding bulls) fathered 41.3% of all 29 offspring sampled in TNP. The infants were assigned to Bruce, Achilles and Aragorn with five, four and three infants, respectively. Out of the remaining 7 bulls, five were not assigned to any infant and two bulls were assigned to one infant each at 80% statistic confidence (Table 4.4 and 4.7). However, using 100% as proportion of males sampled in TNP revealed that the same bulls (Aragorn, Bruce and Achilles) fathered 51.8% of all 29 sampled infants. Aragorn was assigned to 6 infants, Bruce to 5 infants and Achilles to 4 infants. For the remaining 7 bulls, 3 did not sire any infant and one bull sired 3 infants while 3 bulls sired two infants each (Table 4.4 and 4.7).

Table 4.7. Summary of number of offspring assigned to each male.

Bull name	Age	#Offspring (30%)	#Offspring (100%)
Aragorn (TT1)	41	4	6
Bruce (137)	40 – 45	5	5
ATG (50)	35 - 40	0	0
Achilles (40)	35 - 40	3	4
Bombadil (44)	30 - 35	1	2
UNO (28)	30 - 35	0	2
Simon (144)	35 - 40	0	0
Plato (138)	30 - 35	0	3
TT3	40	1	2
Seth (227)	35 – 40	0	0
Amarula(148)*	30 – 35	NA	NA
Capricon(130)*	25 - 30	NA	NA
None (Unresolved)		15	5
Total		29	29

NA = Not applicable, * the samples failed to amplify at all loci, # = number of offspring assigned to each bull at both 30% and 100% (proportion of sampled males in TNP)

CHAPTER FIVE

5.0 DISCUSION

This study was conducted with the aim of determining the genetic diversity and paternity among free-ranging elephants of the Tarangire National Park, Tanzania. This work took the advantage of the vast elephant identification data currently available through the TEP (Foley, 2002) to collect research samples from elephants of known identity for this type of genetic study. The elephant identification database present in TNP is unique in Tanzania since no such long-term elephant studies have been done elsewhere in this country. Sample collection was done using non-invasive approach whereby elephant-specific DNA was obtained from faeces originating from desquamated enterocytes shed out with faeces (Sidransky *et al.*, 1992). The fresh faecal samples from known elephants were carefully obtained by picking the outer most part of the dung to maximise the chances of picking more cells and the samples were preserved in either absolute ethanol or silica as previously described by Wasser *et al.* (1997), Frantzen *et al.* (1998) and Murphy *et al.* (in press).

However, it has been shown that faeces and hair (and other samples obtained non-invasively) used as source of DNA for genetic studies have very low quantity of DNA and in most cases the DNA is degraded (Taberlet *et al.*, 1996, 1999; Taberlet and Luikart, 1999). This has been pointed out as a major drawback for the use of faeces as a source of DNA due to low amplification rate, false alleles, allele dropout and incorrect genotyping restricting faecal DNA to amplification of short mtDNA or nDNA fragments (Taberlet *et al.*, 1996; Frantzen *et al.*, 1998). Thus, appropriate measures including the use of multiple tube approach are used to overcome the above

problems when working with samples with very low DNA quantity (Taberlet *et al.*, 1996; Taberlet *et al.*, 1999)

In this study, faecal DNA extraction was done in duplicate and two different PCR amplifications were done on the two DNA samples extracted separately to test for consistence and repeatability of amplified products. The results obtained were reproducible for most of the samples as shown in Appendix I and the overall amplification as well as genotyping success per locus (81.4 – 91.9%) was high for both silica and ethanol preserved samples. These amplification and genotyping success obtained in this study are comparable to those obtained by Murphy *et al.* (in press) for bear samples but higher than the amplification success of 60% reported for baboons' faeces (Frantzen *et al.*, 1998).

The success of extracting and amplifying DNA from faeces has been shown to depend on the species of the animal, nature of faeces, the amount of inhibitors present in the faeces and size of the genetic markers used (Taberlet *et al.*, 1996; Frantzen *et al.*, 1998). The high amplification and genotyping success obtained in this study might partly be due to the sampling method adopted by picking the outer most part of the dung which is believed to have more cells due to its proximate contact with the intestinal mucosa before being voided. In addition, such results may also be attributed to the use of a two-step faecal DNA extraction procedure using commercial kits (QIAamp® DNA stool kit, QiAgen) to extract and Gene Clean® kit (QiAgen) to concentrate and clean the extracted DNA. This two-step DNA extraction procedure enabled the removal of most of the PCR inhibitors present in faeces.

Moreover, all eight microsatellite marker used in this study were less than 250 bp (Table 3.2) which is the size of microsatellite markers that have been shown to have high amplification success for faecal DNA (Frantzen *et al.*, 1998; Murphy *et al.*, in press).

5.1 Genetic diversity of elephants in TNP

The results obtained from this study have shown that elephants in TNP have high genetic diversity despite severe poaching which greatly decimated most of elephant family groups during the period between 1979 and 1989 (Foley, 2002). High genetic diversity was associated with high number of alleles detected in the population, high number of allele per locus and high heterozygosity values. All 8 microsatellite markers used in this study were highly polymorphic, informative and suitable for parentage analysis as depicted by high PIC, average probability of exclusion and low frequency of null alleles (Marshall *et al.*, 1998; Coltman *et al.*, 1999). Moreover, all loci used in this study did not show any significant departure from HWE and this might indicate that factors that make the population to deviate from HWE are not operating in TNP. Such factors include selection, inbreeding/outbreeding, migration (gene flow from external sources), genetic drift and existence of null alleles.

For any microsatellite marker to be used for parentage analysis, its frequency of null alleles must be less than 0.05 (Marshall *et al.*, 1998). Null alleles most often occur because of mutations in one or both primer binding sites, sufficient to prevent effective amplification of the microsatellite allele or if a sex-linked locus is analysed as if it were an autosomal locus (Marshall *et al.*, 1998). According to Marshall *et al.*

(1998), loci with null alleles at high frequency usually show characteristic patterns of repeated homozygote-homozygote mismatches in known parent-offspring relationships and therefore not suitable for parentage analysis.

The high genetic diversity shown among elephants sampled from TNP might be due to large number of elephants that immigrated into the Park from surrounding areas during the period following the poaching era (Foley, 2002). In addition, the large elephant population size which remained in the Park and free movement of males between TNP and the neighbouring protected areas may have also helped to maintain sufficient gene flow and effective population size (N_e) in Tarangire ecosystem.

A number of studies have been done in other areas to assess the genetic diversity of African elephant using microsatellite DNA markers. One such study done in three national parks in Uganda by Nyakaana and Arctander (1999) reported high genetic diversity in elephant populations severely affected by poaching. Using four microsatellite markers, the same authors obtained a total of 31 allele (n=72) with 6-10 alleles per locus and expected heterozygosity ranging between 0.51 and 0.84. High genetic diversity (44 alleles, 9-14 allele per locus and gene diversity of 0.51-0.72) has also been reported recently in a different study on elephants from East, Central, West and South Africa using six microsatellite markers (Nyakaana *et al.*, 2002).

In another study on savannah and forest elephants, Eggert *et al.* (2002) reported high genetic diversity using both nuclear (4 microsatellite markers) and two mitochondrial

DNA markers. Further studies using 12 microsatellite markers among which included 8 markers used in the present study, reported high genetic diversity in both African and Asian elephants (Comstock *et al.*, 2000). There was higher genetic variation in African than Asian elephants with 3-7 alleles per locus (H_E, 0.258-0.743) and 0-6 alleles per locus (H_E, 0.375-0.765), respectively.

However, studies done in Addo and Kruger NPs in South Africa, reported low genetic diversity in post-bottleneck elephant population using ten microsatellite markers (Whitehouse and Harley, 2001). According to Whitehouse and Harley (2001), a total of 17 alleles, 1.89 mean alleles per locus and H_E of 0.180 were obtained in Addo NP while in Kruger NP they obtained a total of 35 alleles, 3.89 mean alleles per locus and H_E of 0.444. The low genetic diversity in South African elephants was attributed to severe poaching, limited gene flow due to habitat encroachment and founder effects for Addo NP (Whitehouse and Harley, 2001).

The expected heterozygosity of 0.51 to 0.84 and 0.51-0.72 reported by Nyakaana and Arctander (1999) and Nyakaana *et al.* (2002), respectively is comparable to that obtained in this study (H_E, 0.537- 0.817) although the number of alleles detected in TNP was higher than that reported in the above studies. The high number of alleles found in this study might be due to large number of markers used (8) since in the above studies 4 and 6 microsatellite markers, respectively were used. However, the result may also indicate that there is high level of genetic diversity in Tarangire elephants. This is supported by a study by Comstock *et al.* (2000), using 12

microsatellite markers, in which they detected only 59 alleles in Zoo elephants kept in different parts in USA.

5.2 Paternity assignment for Tarangire elephants

In the earlier studies, paternity assignment in wild animal populations was based on exclusion methods to assign a given candidate male to a particular offspring using the available genetic data. Both the candidate parent and the offspring were matched at all loci and paternity would be assigned to a single non-excluded male (Morin *et al.*, 1994). In free-living wild animals, exclusion method was used for parentage testing by Morin *et al.* (1994) in chimpanzee (*Pan troglodytes schweinfurthii)* and Altmann *et al.* (1996) for baboons (*Papio cyanocephalus*). Due to genotypic mismatches that led to high failure of assigning paternity in wild animals, a likelihood method that uses polymorphic codominant markers like microsatellites and an estimated error rate was developed by Marshall *et al.* (1998). The method has been used widely for paternity inference using a web-based computer program, CERVUS (Marshall *et al.*, 1998; Coltman *et al.*, 1999; Gerloff *et al.*, 1999; Slate *et al.*, 2000).

In the present study, appropriate CERVUS simulation parameters were used according to Marshall *et al.* (1998) and only 48% and 83% of the infants were assigned to their potential fathers at 80% and 95% confidence using 30% and 100% as the proportion of sampled males in TNP respectively (Table 4.3 and 4.4). Paternity assignment at 80% confidence means that one out of five paternity assigned is incorrect due to different sources of errors including genotyping errors and

possibly due to high level of genotypic mismatches between the father and offspring (Marshall *et al.*, 1998; Slate *et al.*, (2000). Thus more paternities are expected to be resolved at 80% than 95% confidence at the expense of reduced accuracy due to low delta criteria used with the former.

The resolved paternities described in wild animals are usually low due to difficulty inherent in obtaining samples from all potential fathers (Marshall, *et al.*, 1998). Failure to resolve all paternities in free-living wild animals have been attributed to small proportion of the potential fathers sampled, genotyping errors, mutations and presence of high level of null alleles (Marshall *et al.*, 1998). Thus, 15 (52%) offspring whose paternities were not resolved in the present study when 30% (proportion of males sampled) was used could be due to similar reasons since only 12 out of 40 (30%) potential breeding bulls in TNP were sampled. Moreover, there was high level of genotypic mismatches between offspring and their mothers as well as high error rate as shown in Tables 4.5 and 4.6, which could have lead to failure in paternity assignment for some offspring.

The delta values for resolved paternity tests were high when paternity analysis was done using 30% as proportion of males sampled. The minimum delta values for resolved paternity tests were high at 80% and 95% confidence levels respectively leading to few paternity test resolved (Table 4.3). However, paternity analysis done using 100% as proportion of males sampled utilised very low delta values to resolve paternity tests and the minimum delta value was zero for both 80% and 95%

confidence levels. Due to low minimum delta values, more paternity tests were resolved using 100% as proportion of sampled males in TNP.

Paternity assignment to candidate male when the mother is unknown depends on the sharing of alleles between the candidate males and the offspring at homozygous loci only (Marshall *et al.*, 1998). This results in the reduction of number of loci compared and hence erroneous paternity assignment is more likely to occur than when the mother is known as both homozygous and heterozygous loci are compared. Furthermore, in situations where the mother is known, comparison is made for the number of allele shared by the male, offspring and the mother increasing the accuracy of paternity assignment. Thus, the success in paternity assignment obtained in this study may be attributed to the availability of samples and the corresponding genotypes of known mothers used during paternity analysis.

5.3 Mating success of male elephants

Mating success in male elephants depends on the mating pattern, which involve consort between a female in oestrus and a musth bull (Poole, 1987, 1989b; Foley, 2002). However, other mating patterns such as opportunistic, possessiveness or chase observed in other wild animals like Soay sheep, bonobos and chimpanzee might occur in elephants as well (Poole, 1987, 1989b; Moss, 1988; Coltman *et al.*, 1999; Gerloff *et al.*, 1999; Constable *et al.*, 2001). Assessment of mating success for TNP elephants indicated that 7.5% of the potential breeding bulls were responsible for fathering 41.3 % and 51.7% of all sampled infants at 30% and 100% (proportion of males sampled), respectively. These bulls (Aragorn, Bruce and Achilles) are known

to be the most dominant bulls in TNP (Foley, 2002) indicating that these bulls indeed did most of the mating and resulting in higher breeding success than subordinate bulls. Similar findings have been described in previous studies in Amboseli and Tarangire NPs where dominant males had high mating success based on behavioural data (Poole, 1989b; Foley, 2002). Thus, the success of other mating tactics such as possessive, opportunistic and chase need to be evaluated in order to fully understand the mating success of free living elephant bulls and their corresponding contribution to the genotype of the progeny in the population.

Behavioural studies in elephants have shown that big bulls monopolise most of the matings and come in musth at the time when most of the females are likely to come in heat (Poole, 1987, 1989b, 1989c; Foley, 2002). These big bulls stay in musth for a longer time, can protect females in heat against harassment of young bulls and thus are preferred by mature and experienced females. In addition, female elephants show synchronised oestrous and thus dominant bulls with musth period that coincide with optimal breeding period of most females in the population are likely to sire more infants. Thus, the results obtained in this study whereby three big bulls were assigned to 41.3 % and 51.7% of all sampled infants at 30% and 100% respectively are in agreements with the above observations.

In other wild animals, paternity inference has also been determined using likelihood method. For paternity analysis done in Soay sheep (*Ovis aries*), Coltman *et al.* (1999) were able to assign 61.9% of the lambs (n=365) to their fathers at 80% statistic confidence. The same study revealed that the mating success of the male was

positively correlated to its general fitness since adult males (32% of all breeding rams) sired 62.5% of all infants for cohorts born in three consecutive years.

Studies in chimpanzee (*P. troglodytes schweinfurthii*) at Gombe NP by Constable *et al.* (2001) reported the successful assignments of all 14 sampled infants to their potential fathers. Like in other wild animals, the same study revealed that alpha and high-ranking males fathered 50% of all sampled infants in Kasakela community. Similar findings were also reported in baboons (*P. cyanocephalus*) by Altman *et al.* (1996), whereby one dominant male accounted for 44% of 63 surviving infants born in the population for a period of 11 years in Amboseli NP. Furthermore, studies on bonobos by Gerloff *et al.* (1999) showed that reproductive success was biased in favour of high-ranking males.

The findings from this and other studies on different wild animal species suggest that dominant males monopolise most of the mating and hence attain the highest reproduction success. However, for elephants, the potential for female choice might be an alternative explanation for the bias in paternity distribution (Poole, 1989b; Gerloff *et al.*, 1999). This is because adult and experienced female elephants have been shown to exercise choice by mating with adult bulls in musth (Poole, 1989b). Though the effect of female choice for mature bulls during mating on the breeding success of individual bull elephant was not evaluated in the present study, such ability may possibly be the reason for the high reproductive success reported in this study for the dominant males.

5.4 Ambiguous paternity assignments

At 30% simulation, T160 (Lynne) female offspring was assigned to T44 (Bombadil) and T207 (Desdemona) offspring was assigned to TT3 as their possible fathers at 80% confidence (delta = 1.387) and TT3 (delta = 1.205), respectively. T160 and T207 were assigned to the same bull with the same delta values as above at 95% confidence when 100% simulation (as proportion of sampled males) was used (Table 4.4). According to Foley (personal communication), the two female offspring are currently aged over 20 years, Bombadil (T44) is just over 30 years old now and TT3 died in the year 2000 at about 40 years of age (Table 4.7). At the time T160 and T207 were born, these two bulls were too young to have successful mating and impregnate an adult female elephant in the wild. Based on the age differences, these females are thus unlikely to have any father in TNP since the oldest bull in TNP (Bruce-T137), aged about 42 years old now would have been too young to mate successfully at the time of their birth. Thus, these two paternity assignments were considered to be questionable and ambiguous based on the field data on the ages of both offspring and the bulls.

Furthermore, T160 could have been erroneously assigned to Bombadil (T44- bull) due to low numbers of loci (4 loci) genotyped for T44 and high genotyping errors on T160 and her mother's (T156) genotypes due to 3 mismatches (Table 4.5). On the other hand, T207 seem to be closely related to TT3 due to high number of alleles shared between them (7 alleles at 8 loci) and the high number of alleles shared between her mother (T208) and TT3 (5 alleles at 8 loci)(Appendix 1). The exact nature of relatedness between the above animals will possibly be resolved by

genotyping more loci for T44 and kinship analysis which was not done in the current study. The kinship analysis on the animals used in the present study is currently being performed and will be reported elsewhere.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the findings of the present study, the following conclusions can be drawn

1. This study revealed that Absolute Ethanol and silica were good preservatives of faecal sample from which good quality DNA was obtained. The faecal DNA was successfully amplified and had 81.4 to 91.9% (mean, 87.9%) success of scoring individual genotypes using 8 microsatellite markers.
2. The 8 microsatellite markers used showed a high level of polymorphism which enabled to show that elephants in TNP have high genetic diversity apart from severe poaching which occurred in the Park between 1979 and late 1980s.
3. The genotype data from eight microsatellite markers enabled to assign 14 (48%) and 24 (83%) out of 29 offspring to their fathers at 80% confidence for 30% and 100% simulation, respectively.
4. Assessment of mating success among bulls in TNP revealed that 7.5% of all potential breeding bulls were fathers of majority of offspring sampled from TNP. This type of breeding success poses a danger of inbreeding in small population or in populations that were severely affected by poaching.
5. This study provides for the first time an important genetic database that has wide applications in future studies of African elephants in TNP and other conservation areas in Tanzania. The data obtained will be used to assess the genetic relatedness of elephants residing in the park as well as assist in providing an insight on the effects of poaching on the current elephant social structure in TNP.

6.2. Recommendations

The results obtained from this study provide valuable information, on the genetic diversity and paternity among free-ranging elephants in TNP. This information is the first of its kind in Tanzania and will prove extremely useful in understanding and conserving the endangered African elephants in Tanzania as well as in other range states in Africa. However, addition information on the following aspects is inevitably required for proper utilisation of these findings:

1. Sampling more breeding bulls in TNP is vital so as to enable full assessment of the mating success of the free-living male elephants.
2. More samples need to be obtained and analysed from more elephant families and more microsatellite markers should be used in order to utilise the available data to establish the genetic relatedness among elephants in TNP.
3. There is a need to conduct kinship and paternity studies on a relatively less poached elephant population like those residing in Amboseli National Park. This will provide comparative baseline data on the kinship and mating success of free-living African elephant based on DNA analysis. Such information will be useful in assessing the effect of poaching pressure on the intactness of elephant social structure in TNP and other populations.
4. Behavioural methods through which both male and female elephants avoid inbreeding and the corresponding level of inbreeding in both poached and unpoached population need to be objectively determined. This will possibly give a better estimation of the effects of poaching on elephants in general and particularly on the breeding success of males of different ages in the population.

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APPENDICES

Appendix 1: List of 86 elephants sampled from TNP and their corresponding genotypes for 8 microsatelite markers.

ID#	NAME	GRP	FH102-1	FH102-2	FH103-1	FH103-2	FH126-1	FH126-2	FH153-1	FH153-2	FH19-1	FH19-2	FH48-1	FH48-2	FH67-1	FH67-2	FH94-1	FH94-2
177	ADINA '96 (F)	A	177	179	151	153	100	108	167	173	189	193	172	176	95	105	226	226
180	ACACIA	A	177	179	149	149	94	98	167	167	185	189	172	172	97	103	226	226
183	ADRIA	A	177	179	151	153	98	108	167	167	189	193	172	176	95	103	224	226
184	BIG MAMA	A	177	179	149	151	108	112	167	177	185	185	168	168	97	97	226	230
238	ASHLEY	A	177	181	145	147	94	106	161	169	185	193	168	168	91	103	226	230
242	ANDY	A	177	177	147	151	94	106	167	169	185	185			91	95	226	226
244	AMY (F) 99	A	177	181	151	151	106	106	169	173	193	195	174	174			226	226
176	ADINA	A	179	181	145	145	94	94	169	169	187	193	168	168	103	105	230	230
178	ANNETTE	A	177	177	149	151	106	106	165	167	185	193	168	168	95	95	226	226
179	AMY	A	177	179	151	151	106	106	167	169	193	193	172	174	97	105	224	226
181	ALEXIS	A	179	181			106	108	165	167	183	193	168	168	95	97	224	230
182	ALLEGRA	A	177	179	149	151	96	108	165	167	189	189	168	172	97	103	226	226
236	ADDO	A	177	181	145	151	94	98	167	169	185	193	168	172	95	103	226	230
237	ALISON (F) '98	A					104	106	157	169					95	103	224	226
239	ALISON	A	177	179	151	151	106	108	157	167	189	193	172	178	95	105	226	226
240	ADINA (F) '98	A	177	179	145	151	94	94	167	169	187	193	168	168	103	103	224	230
241	ADRIA (F) '00	A	177	179	151	151	96	108	167	167	185	193	168	172	95	95	226	226
243	ALEXIS (F) 96	A	179	179	149	149	98	106	167	167	189	193	168	178	95	103	226	230
245	ADDO (F) 00	A	177	179	147	151	98	104	167	169	185	185	168	172	95	95	226	230
229*	CONNIE	C																
230	CONCHA	C			149	151												
231*	CRISCINT	C																
272	CSP (F4)	CSP	179	179	151	151	94	106	167	173	187	193	168	168	95	105	226	230
274	CSP (F5)	CSP			151	151									95	97		
281	CSP (F7)	CSP	177	177	145	151	106	106	161	167	185	187	168	172	95	105	226	230
282	CSP (F7)	CSP	177	179	151	151	104	112	167	167	185	193	168	172	95	97	226	226

ID#	NAME	GRP	FH102-1	FH102-2	FH103-1	FH103-2	FH126-1	FH126-2	FH153-1	FH153-2	FH19-1	FH19-2	FH48-1	FH48-2	FH67-1	FH67-2	FH94-1	FH94-2
273	CSP (F5)	CSP	179	179	145	151	98	108	163	171	185	187	168	172	97	99	226	230
275	CSP (F5)	CSP	177	179											99	103	226	226
283	CSP (F7)	CSP	177	179	151	151	104	108	167	167	185	189	168	168	95	97	226	226
280	CSP (F6)	CSP	177	179	145	151	106	108	165	167	185	189			95	97	226	226
207	DESDEMONA	D	177	179	145	151	98	108	167	175	187	193	168	168	95	97	224	226
208	FANG	D	177	187	151	151	98	108	169	175	187	193	168	168	95	97	226	226
211	VAMPIRE	D	177	177	145	151	94	106	161	167	185	187	170	172	95	97	224	226
193	ISABEL '97 (F)	I	177	177	149	151	98	98	167	173	187	193	168	174	103	103	224	226
196	INESITA	I	177	179	145	147	104	108	165	167	193	193	168	172	95	99	224	226
198	INES '99 (F)	I	179	179	151	151	104	108	167	167	189	193	168	172	95	99	224	224
200	ISABEL '00 (F)	I	177	177	147	151	98	106	165	167	193	193	168	168	95	97	224	226
234	ISSELLE	I	179	181	145	149	104	106	169	171	187	189	168	172	97	97	224	226
194	INES '96 (F)	I	177	177	145	153	98	108	167	169	183	193	172	172	99	103	226	230
195	ISELLE 96 (M)	I	179	181	149	153	98	104	157	171	185	189	168	172	105	105	226	226
197	ISELLE '99 (M)	I	181	183	145	151	106	112	161	171	187	193	172	174	95	97	226	226
199	IVAN	I	177	181	151	151	106	112	167	167	187	193	168	172	103	105	224	226
232	INES	I			145	151	108	108	163	167					97	99	224	226
233	ISABEL	I	177	177	149	151	98	112	167	175	185	187	168	168	97	103	224	226
156	LORRAINE	L	177	187	145	147	94	106	165	173	185	187	168	172	91	105	226	230
158	LAKINI	L	177	177	145	151	98	106	157	165	187	193	172	172	91	103	226	226
154	LAUREN	L	177	187	147	147	94	94	165	171	185	193	172	172	97	105	226	226
155	LEA	L	181	181	145	151	94	106	167	171	185	193	168	168	105	105	224	226
157	LAURENS 00 (M)	L	179	187	147	151	94	106	161	171	185	185	168	172	103	105	226	226
160	LYNNE	L	177	177	149	153	94	104	161	167					95	97	226	226
161	LYNNE '99 (M)	L	177	179	145	150	94	94	167	167	196		170	178	95	103	226	226
162	LYNNE '96 (M)	L	177	177	151	151	98	104	167	169	183	185	170	172	95	103	226	230
218	LEDA '99 (F)	L	177	177			98	106							91	103	226	226
219	LEDA	L	177	181	151	151	94	106	169	171					91	97	226	230
256	X-LEY	X	177	181	149	151	106	108	169	183	185	185	168	172	82	82	226	226
227	SETH	MALE	179	179	151	151	106	112	163	173	183	187	172	172	97	99	224	226

ID#	NAME	GRP	FH102-1	FH102-2	FH103-1	FH103-2	FH126-1	FH126-2	FH153-1	FH153-2	FH19-1	FH19-2	FH48-1	FH48-2	FH67-1	FH67-2	FH94-1	FH94-2
144	SIMON	MALE	177	179	151	151	106	112	165	169	185	189	168	172	95	95		
40	ACHILLES	MALE	177	179	145	153	94	98	165	169	183	187	172	172	93	95		
148	AMARULA	MALE																
28	UNO	MALE	177	179	151	151	106	109	167	167	189	189	172	172	95	99		
44	BOMBADIL	MALE	177	177	149	151					189	189	168	172	95	97		
138	PLATO	MALE	177	181	151	151	104	106	167	173	185	189	168	172	103	103	226	226
130	CAPRICON	MALE																
137	BRUCE	MALE	177	177	147	151	98	106	161	167	185	193	168	170	91	103	226	226
50	ATG	MALE	177	179	149	151	106	112	165	169	185	187	168	172	91	95	230	230
TT3	TISSUES	MALE	177	181	145	145	106	108	165	183	193	195	156	172	95	95	224	226
TT1	TISSUES	MALE	177	179	151	153			157	157	185	193	172	174	103	105	226	226
265	SSP (F2)-	SSP	177	179	149	151			165	171	183	187	172	178	97	103	230	230
266	SSP (F2)	SSP	177	177	145	151	106	106	167	171	185	187	170	178	99	103	224	224
267	SSP (F2)	SSP	177	177	147	151	94	108	167	171					99	103	226	230
268	SSP (F2)	SSP	177	177	149	151	94	106	161	171	185	187	172	178	95	99	224	226
269	SSP (F3)	SSP	177	177	145	151	98	106	165	165	185	193	168	168	99	103	226	226
270	SSP	SSP	177	181	145	151	106	106	163	167	183	193	168	172	99	105	226	230
202	TABBY	T	177	187	149	151	98	108	167	169	187	193	168	172	95	97	224	224
221	TIA	T	177	177	145	151	108	108	157	167	187	193	172	174	95	105	224	226
165	VANESSA	VA	177	179	145	151	108	108	167	167	183	193	168	168	97	97	226	226
166	VALERIE	VA	179	181	145	151	94	108	169	171	185	187	168	168	95	97	224	226
167	VANGUARD	VA	177	177	145	151	98	98	167	167	185	193	168	174	95	97	226	226
213	WILLOW	W	177	181	145	151	116	116	167	167	193	193	168	172	95	95	224	224
212	WISTERIA	W	177	179														
214	WHISPER	W	177	177	151	153	112	112	161	167	185	193	170	172	99	105	226	230
164	X-TUSK	X	177	181	149	151	106	108	167	169	185	189	168	174	83	85	226	226
255	SLIT EAR	X	179	179	151	151	98	106	167	169	185	193	172	172	101	103	226	230
251	X-ROAD	X	177	177	145	149	96	98	167	169	185	185	172	172	95	103	224	226
163	X-TUSK '99 (F)	X	177	177	151	153	98	104	169	173	183	185	172	176	97	97	226	226
257	SLIT EAR '96 (M)	X	179	179	151	153	98	106	169	173	187	193	172	174	103	103	226	230

Appendix 2: Paternity assignment summary 30% as proportion of males sampled in TNP

O- ID	O LT	KP ID	KP-LT	O-KP LCD	O-KP LM	Prob. NE	CP ID	CP LT	O-CP LCD	O-CP LM	O-KP-CP LCD	O-KP-CP LM	LOD	Delta	Confidence
243	8	181	7	7	0	0.000	28	7	7	2	6	3	-1.127	0.000	
241	8	183	8	8	0	0.003	28	7	7	2	7	3	0.594	0.290	-
237	4	239	8	4	0	0.006	40	8	4	1	4	2	0.247	0.247	-
182	8	180	8	8	1	0.014	44	5	5	0	5	0	2.118	0.959	-
244	7	179	8	7	0	0.000	138	8	7	2	7	2	2.396	0.642	-
176	8	236	8	8	0	0.000	40	8	8	2	8	2	2.151	2.151	+
240	8	176	8	8	0	0.007	44	5	5	2	5	2	-2.161	0.000	
242	7	236	8	7	0	0.002	137	8	7	0	7	0	4.988	4.988	*
245	8	236	8	8	0	0.002	144	8	8	1	8	2	-0.489	0.000	
177	8	241	8	8	0	0.000	TT1	7	7	1	7	3	1.380	1.380	+
238	8	176	8	8	0	0.000	137	8	8	0	8	0	6.966	6.966	*
207	8	208	8	8	0	0.009	TT3	8	8	2	8	3	1.205	1.205	-
199	8	233	8	8	0	0.004	138	8	8	1	8	2	0.273	0.273	-
193	8	233	8	8	0	0.001	TT1	7	7	1	7	1	2.489	1.764	+
200	8	233	8	8	1	0.004	137	8	8	1	8	3	1.547	0.526	-
195	8	234	8	8	1	0.001	TT1	7	7	0	7	1	3.869	3.869	*
197	8	234	8	8	0	0.000	TT1	7	7	3	7	3	0.346	0.346	-
196	8	232	5	5	0	0.001	TT3	8	8	0	5	2	-0.916	0.000	
194	8	232	5	5	0	0.000	40	8	8	1	5	1	5.363	4.912	*
198	8	232	5	5	0	0.004	28	7	7	1	4	1	3.351	2.150	+
160	6	156	8	6	3	0.106	44	5	3	0	3	2	1.387	1.357	+
161	7	160	6	6	1	0.005	TT1	7	6	3	5	2	1.119	1.119	-
162	8	160	6	6	1	0.004	40	8	8	1	6	2	2.210	2.210	+
154	8	156	8	8	0	0.001	TT1	7	7	2	7	3	-0.562	0.000	
157	8	154	8	8	0	0.002	137	8	8	1	8	1	1.811	1.374	+
158	8	156	8	8	0	0.002	TT1	7	7	0	7	0	2 529	1.700	+
155	8	219	6	6	1	0.008	TT3	8	8	3	6	2	2.483	2.483	+
218	4	219	6	4	0	0.085	137	8	4	0	4	0	2.589	1.582	+
256	8	164	8	8	1	0.004	138	8	8	2	8	2	1.497	1.101	-

Appendix 3: Paternity assignment summary with 100% as proportion of males sampled in TNP

O- ID	O LT	KP ID	KP-LT	O-KP LCD	0-KP LM	Prob. NE	CP ID	CP LT	O-CP LCD	O-CP LM	O-KP-CP LCD	O-KP-CP LM	LOD	Delta	Confidence
243	8	181	7	7	0	0.000	28	7	7	2	6	3	-1.127	0.000	
241	8	183	8	8	0	0.003	28	7	7	2	7	3	0.594	0.290	*
237	4	239	8	4	0	0.006	40	8	4	1	4	2	0.247	0.247	*
182	8	180	8	8	1	0.014	44	5	5	0	5	0	2.118	0.959	*
244	7	179	8	7	0	0.000	138	8	7	2	7	2	2.396	0.642	*
176	8	236	8	8	0	0.000	40	8	8	2	8	2	2.151	2.151	*
240	8	176	8	8	0	0.007	44	5	5	2	5	2	-2.161	0.000	
242	7	236	8	7	0	0.002	137	8	7	0	7	0	4.988	4.988	*
245	8	236	8	8	0	0.002	144	8	8	1	8	2	-0.489	0.000	
177	8	241	8	8	0	0.000	TT1	7	7	1	7	3	1.380	1.380	*
238	8	176	8	8	0	0.000	137	8	8	0	8	0	6.966	6.966	*
207	8	208	8	8	0	0 009	TT3	8	8	2	8	3	1.205	1.205	*
199	8	233	8	8	0	0.004	138	8	8	1	8	2	0.273	0.273	*
193	8	233	8	8	0	0.001	TT1	7	7	1	7	1	2.489	1.764	*
200	8	233	8	8	1	0.004	137	8	8	1	8	3	1.547	0.526	*
195	8	234	8	8	1	0.001	TT1	7	7	0	7	1	3.869	3.869	*
197	8	234	8	8	0	0.000	TT1	7	7	3	7	3	0.346	0.346	*
196	8	232	5	5	0	0.001	TT3	8	8	0	5	2	-0.916	0.000	
194	8	232	5	5	0	0.000	40	8	8	1	5	1	5.363	4.912	*
198	8	232	5	5	0	0.004	28	7	7	1	4	1	3.351	2.150	*
160	6	156	8	6	3	0.106	44	5	3	0	3	2	1.387	1.357	*
161	7	160	6	6	1	0.005	TT1	7	6	3	5	2	1.119	1.119	*
162	8	160	6	6	1	0.004	40	8	8	1	6	2	2.210	2.210	*
154	8	156	8	8	0	0.001	TT1	7	7	2	7	3	-0 562	0.000	
157	8	154	8	8	0	0 002	137	8	8	1	8	1	1.811	1.374	*
158	8	156	8	8	0	0.002	TT1	7	7	0	7	0	2.529	1.700	*
155	8	219	6	6	1	0.008	TT3	8	8	3	6	2	2.483	2.483	*
218	4	219	6	4	0	0.085	137	8	4	0	4	0	2.589	1.582	*
256	8	164	8	8	1	0.004	138	8	8	2	8	2	1.497	1.101	*

O – Offspring, CP-candidate parent, KP- Known Parent, LT-Loci typed, LCD-Loci compared, LM-Loci mismatching, Prob. NE - Probability of non-exclusion, LOD- log (to base e) of the product of the likelihood ratio.

‡ Confidence levels are displayed as follows: *: Strict confidence (95%); +: Relaxed confidence (80%); -: Most likely and any candidate parent that is not most likely is shown with a Delta score of zero, and the confidence column contains an empty string.