MITOCHONDRIAL DNA VARIATION OF EASTERN AFRICAN LION (*PANTHERA LEO MELANOCHAITA)* POPULATIONS IN KENYA

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DECLARATION

This thesis is my original work, and to the best of my knowledge, has not been submitted for examination for the award of degree in any other institution of higher learning

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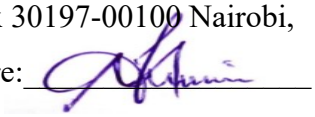
Signature:  Date: 05-05-2021

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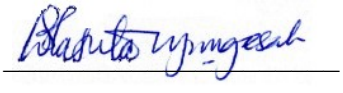
Signature:  Date: 06-05-2021

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DEDICATION

This MSc. Thesis is dedicated to my lovely wife Beth Irungu, our two daughters Mercylily Irungu and Victoria Irungu and my lovely mother Sarah Njeri Maguta for their love, moral support, encouragement and sincere prayers for me during my study and research work. I also dedicate this work to my late father Mr. Daniel Maguta for every effort he made in my academic life.

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LIST OF ABBREVIATIONS

CITES: Convention for International Trade on Endangered Species

IUCN: International Union for Conservation of Nature

IFAW: International Fund for Animal Welfare

NCBI: National Center for Bioinformatics Information

KWS: Kenya Wildlife Services

KVD: Kenya Veterinary Department

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

rRNA: Ribosomal RNA

tRNA: Transfer RNA

CDS: Coding sequences

CYTB: Cytochrome B

COX: Cytochrome Oxidase

NADH: Nicotinamide Adenine Dinucleotide Dehydrogenase

ND: NADH Dehydrogenase

ATP: Adenine Triphosphate

HVR: Hyper-Variable Region

MT: Mitochondria

Min: Minutes

Sec: Seconds

mM: MilliMolar

°C: Degree Celcius

V: Voltage

ng: Nanogram

mg: Milligram

Ml: Milliliter

FIV_{ple}: Panthera leo specific Feline Immune Deficiency Virus

F_{IS}: Inbreeding coefficient

F_{ST}: Coefficient of differentiation

SSC: Species survival commission

CSG: Cat specialist group

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ABSTRACT

The Eastern African lion population forms a major part of the wildlife ecosystem with key contributions in the economy of the country through ecotourism, employment, cultural values and maintenance of biodiversity. Less than 2000 lions are estimated to live in Kenya, a decline of over 90% in the last 100 years. Due to the increased human pressure and other numerous underlying factors on the ecosystems, the lion populations is unstable and at risk of local extirpation. Proper knowledge of the aspects of their genetic structure is a fundamental step toward improved management and conservation of lions. Yet there is insufficient genetic information in most of the Kenyan lion populations. The aim of this study was to disclose the mitochondrial D loop polymorphisms, genetic variations and maternal lineages of *Panthera leo melanochaita* populations in Kenya by sequencing the mitochondrial D-loop region (932bp in length). The 932 bps mitochondrial D-loop region studied exhibited 282 polymorphic sites (S) with 211 singleton variable sites and 71 parsimony informative sites. This region also exhibited low mean genetic diversity (within subpopulation and in entire population) (0.052), low nucleotide diversity (pi=0.02103) and substantial haplotype diversity (Hd=0.964). The northern frontier lion population showed high polymorphism, mean genetic diversity and high nucleotide diversity than the southern frontier lion population. Haplotype analysis indicated 41 haplotypes with 4 shared haplotypes and 37 haplotypes that were unique. Phylogenetic analysis indicated a single monophyletic clade, one maternal lineage and six haplogroups with five haplogroups that were resolved and were distinct.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

The global lion population experienced dramatic decline and extinction that left Africa and Asia's lion populations as the only lion populations (IUCN, 2006b; Bauer et al., 2016). Similar decline has been experienced regionally and locally with negative impacts on the global, regional and local economy (Okech, 2011; Ripple et al., 2014; Williams et al., 2017). The former taxonomic classification of felidae recognized the remaining two lion populations as the African lion (*Panthera leo leo*) and the Asiatic lion (*Panthera leo persica*) populations (Barnett et al., 2014; Bertola et al., 2016; Kitchener et al., 2017). The African lion population live in Sub-Saharan Africa and the Asiatic lion population in India's Gir forest estimated to be 1,400 km^2 (IUCN, 2006b; Riggio et al., 2013). To adopt the best conservation strategies for the remaining lions in Africa and Asia, the classification was reviewed and revised through the recommendation of the International Union for Conservation of Nature (IUCN) task force for cat classification, Commission for Species Survival (SSC) and a specialist group for cats (Barnett et al., 2014). The revised classification recognized *Panthera leo melanochaita* living in Southern and Eastern Africa and *panthera leo leo* subspecies in Asia, North Africa, Central and West Africa (Kitchener et al., 2017).

This review was due to a number of phylogenetic studies done on the mitochondrial DNA molecular markers including the mtDNA D loop, cytochrome b, 12S and 16S rRNA on the African lions that identified the eastern and southern African clade and the Asiatic lions, North African lions, west African lions and Central African lions as two separate lion clades (Antunes et al. 2008; Barnett et al., 2014; Bertola et al., 2016). Genetic variations of the

mtDNA regions have also been used to define the lion populations in to different groups including the modern, cave and American lions (Antunes et al., 2008; Dubach et al., 2013). The Eastern Africa lion subspecies in Kenya are found in the northern and the southern rangelands that define its wildlife protection and conservation units. Majority of the remaining lion population in Kenya are known to live in the Maasai Mara and Tsavo ecosystems. There are small parched populations in the northern part of Kenya, Samburu, Laikipia, Kajiado, Machakos, Coastal Kenya and in some conservancies in Wajir that are communally managed.

Conservation of wildlife in Kenya especially the big five species including lions is highly embraced because it has key contributions to the country's economy. The highly growing human population in Kenya has negatively impacted on wildlife conservation due to anthropogenic developments like human settlements, road construction and large-scale crop farming in wildlife dispersal areas. Because of these challenges together with other underlying factors, the current lion population in Kenya is experiencing dramatic decline with far reaching negative effects on the country's economy. Conservationists has estimated that Kenya loses a 100 lions per year, while the current lion population stands at approximately 2,000 lions or less, the population is feared to be at risk of extinction within the next 15 to 20 years (Daily Nation Newspaper, 3rd March, 2019). This study was carried out in selected areas of ecosystems in the Northern and Southern rangelands in Kenya. The sampled areas in the northern frontier included Maralal, Marsabit, Waso, Lewa, Isiolo and Garissa while those sampled in the southern frontier included Nairobi National Park, the Maasai Mara National Reserve and its neighboring privately owned conservancies including Mara north, Naboisho, Olare Motorogi, Olare Naboisho and Ol Kinyei.

1.2 Problem statement

Although mitigation measures have been put in place especially the establishment of agencies like lion guardians and regional conservation strategies formulated through several conventions (Frank, 2011; Watts, 2016), Kenya still records significant decline in lion population (Kenya Wildlife Service, 2008) due to rampant killing (Hazzah et al., 2017; Ontiri et al. 2019) and several other underlying factors described in the literature review in this study. Understanding the lion population dynamics and genetic structure of the remaining lion population is very key to wildlife conservation in order to adopt the best conservation practices of genetic rescue (IUCN, 2018). While studies on genetics have been done on a number of lion populations across certain parts of Africa (Dubach et al. 2005, 2013; Barnett et al. 2006b; Bertola et al., 2015), Kenya is currently conducting scientific survey on lions to establish population estimate, however there is insufficient information on the genetic status of Kenya's lion population (IUCN, 2006a, b; KWS, 2009).

1.3 Justification of study

Panthera leo melanochaita plays a major role as the country's economic pillar through local and international tourism, employment opportunities, cultural values and biodiversity maintenance. Through predation, it also helps in balancing the ecological communities (Okech, 2011; Ripple et al., 2014; Williams et al., 2017). Despite these benefits, the sub species population in Kenya is rapidly declining due to pressures emanating from human population growth leading to anthropogenic developments, habitat loss, human-wildlife conflict, prey loss and diseases. Consequently, the remaining lion populations are highly isolated and fragmented and their future persistence is uncertain (Winterbach et al., 2013;

Trinkel and Angelici, 2016). The National Wildlife Management and Conservation Policy (2017) recommends conservation of wildlife based on scientific data. Therefore understanding the genetic structure is necessary to formulate conservation and management decisions and actions that would help save this population from local extinction.

1.4 Research question

Do the northern and the southern lion populations in Kenya present differences in genetic polymorphism, genetic variations and phylogenetic relationships

1.5 Hypothesis

The two lion populations in the southern and the northern frontier of Kenya are genetically similar with low genetic diversity.

1.6 Objectives

1.6.1 Main Objective

- ❖ Evaluate the mitochondrial DNA polymorphisms of Eastern African lion populations in Kenya

1.6.2 Specific Objectives

1) Estimate the genetic variations of the Kenyan lion subpopulations by the use of mitochondrial D-loop genetic marker.
2) Determine the maternal lineages of fragmented lion populations in Kenya to establish their phylogenetic relationships.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Panthera leo

The *Panthera leo* is the second largest felid species from tiger (*Panthera tigris*) (Murphy and Macdonald, 2010). In Africa's Savannah ecosystem, it is the top terrestrial and apex predator with a top-down biodiversity population control of the ecosystem (Hayward et al., 2007; Williams et al., 2017). It is well known to be a key species among *panthera genus* big five roaring cats (Caro and Riggio, 2014). In human culture, it has played a crucial role (Patterson, 2007) in maintaining a strong and rich biodiversity among others (Schmitz et al. 2010; Estes et al. 2011). Lion also belongs to the seven species that make up the African large carnivore guild among others. These other species include the leopard (*Panthera pardus*), Spotted hyena (*Crocuta crocuta*), Stripped hyena (*Hyaena hyaena*), Brown Hyena (*Hyaena brunnea*), Cheetah (*Acinonyx Jubatus*) and Wild dog (*Lycaon pictus*) (Dalerum et al., 2008). According to the IUCN assessment, a decreasing trend characterize all these species except the spotted hyena with the lowest conservation concern (IUCN, 2015). Among the seven African guild species, the lion plays the biggest role in functional ecological diversity (Dalerum, 2013). It is classified as vulnerable under Criterion A2abcd according to the IUCN Red List of Threatened Species (IUCN, 2018; Tuqa et al., 2014). Lions have also been on the list for consideration for endangered species (IFAW, 2011).

Panthera leo exhibits a variety of distinct phenotypic or morphological variations. This is also exhibited **i**n other *Panthera genus* including *Panthera tigris*, snow leopard (*Panthera uncia)*, Jaguar *(Panthera onca)* and leopard (*Panthera pardus)* (Hallgrimson, 2002; Dubach et al., 2005). The major variations exhibited includes variations in body size, skull features, coat

color and thickness, retention of juvenile spots and the presence or absence of mane in males (Mazak, 2010; Hollister, 2011).

Lions are the most social species living in family units called prides. Each family pride consists of 2 to 18 related females and 1 to 7 males that migrate into the pride from different prides (Mosser and Packer, 2009; Kotze and Robynne, 2016). Prides are characterized by high rate of male lion dispersal patterns than female lion dispersal (Bauer et al., 2016; Verschueren, 2017). The male lion offspring show sex-biased dispersal where they leave their natal family (Mosser and Packer, 2009) and disperse to long distances at puberty while female lions remain in their birth home for life (Verschueren, 2017). High rate of male biased dispersal patterns in lions may result in different distributions of genetic variations among populations (Tende et al., 2014 a, b). In some cases, the female lions may also leave their natal pride to establish a new pride close to their natal range, which often includes part of their old range (Dubach et al., 2005; Kotze and Robynne, 2016). A genetically viable population need to have 50 or more prides of free ranging lions (Bjoerklund, 2003; Bauer et al., 2016). Their home ranges are largely influenced by prey-base abundance, clean water (Celesia et al., 2009), habitat quality, social factors, sex, season, presence of livestock, territoriality and group size (Tumenta et al., 2013; Tuqa et al., 2014; Loveridge et al., 2009, 2018).

2.2 Lion Conservation Units (LCUs)

It is estimated that approximately 209,000 wildlife protected areas exist globally, which covers about 15.4% global land mass (Juffe-Bignoli et al., 2014). Out of the total land mass in Africa, 14.7% is estimated to contain protected areas owned by the states. Additionally, a number of countries have huge size of land set aside as wildlife network connectivity zones. Among these states include Zambia with 38% and Tanzania with 32% of their land being used as

wildlife dispersal corridors. Tanzania has set aside approximately 90,000 Km^2 in Selous Game Reserve. Zambia has also set aside 66,000 Km^2 in Kafue National Park while Angola has set aside 84,000 Km^2 in Luengue-Luiana-Maringa complex as lions management areas (Barnett et al., 2006b). Other countries including Southern Africa have huge land mass as Trans-Frontier Conservation Areas (TFCAs). For example, Kavango-Zambezi TFCA covering approximately 520,000 Km^2 (Fabre, 2012; European Commission, 2015; McKinnon et al., 2016).

The African Sub-Saharan Savannah habitats cover approximately 13.5 million Km^2. Out of this the African lion population range covers approximately 3.0 to 3.5 million Km^2. This is 17% to 25% of their historic range and is considered suitable habitats for lions conservation (IUCN, 2006 a, b; Ripple et al., 2014). The IUCN enlists about 1.08 million Km^2 as protected land with large National Parks, Game Reserves and wilderness lands in savannahs (Riggio et al., 2013; Watts, 2016). The African continent was estimated to have 86 Lion Conservation Units (LCUs) (IUCN, 2006a; Valley and Valley, 2014) established using *Panthera onca* conservation model (Sanderson et al., 2002; Dubach et al., 2013). They cover an estimate of 3,163, 260 Km^2 in line with the estimated 3,390, 821 Km^2 suitable habitats for lion conservation (Riggio et al., 2013). Out of these LCUs, approximately 588,000 Km^2 have been set aside as trophy hunting zones for lion harvesting (Lindsey et al., 2013a). The remaining suitable habitats being fragmented with parched lion areas characterized by poorly protected and declining populations (Newmark, 2008). On the other hand, the Eastern and Southern Africa were estimated to have 66 lion conservation areas covering 61% of their range (Sanderson et al. 2002; Dubach et al. 2013). The Wildlife Conservation Society (WCS) estimate about 67 lion conservation areas of extant populations covering 2.4 million km^2 (17%) of historical range and 25% of African Savannah (Bauer et al., 2016).

The Eastern and Southern Africa supports majority of the remaining living lions (Bauer and Van Der Merwe, 2004; Dubach et al., 2013) including all the ten lion strongholds in Africa (Dubach et al., 2013; Riggio et al., 2013). In West and Central Africa, most LCUs no longer contain lions (Henschel et al., 2010; 2014). Over half of the total lion range totaling 1,811087 km^2 (52%) was classified as extinct while the rest estimated to be 1, 654, 375 km^2 has been classified as extant (Figure 2.3) (Riggio et al., 2013). Kenya constitutes approximately 7,194 million hectares of protected areas (PAs) out of the total 58,037 million hectares total land mass in the country (Tolvanen, 2004; Kameri-Mbote, 2005). This approximate to 12.3% designated protected areas as outlined by the IUCN (Bauer et al., 2016). Some of the National Parks in the category I and II of the IUCN protected areas in the country cover an estimated area of 3,432 million hectares (Tolvanen, 2004; Kameri-mbote, 2005).

2.2.1 Importance of LCUs

Protected areas have a crucial role in keeping safe and protecting the integrity of wildlife habitats and biodiversity (Geldmann et al., 2013). They also interrupt the natural patterns of wildlife migration, dispersal and gene flow along their dispersal corridors (Somers and Hayward, 2012; Trinkel et al. 2013; Dubach et al. 2013). Establishment and proper management of lions' conservation units requires a comprehensive knowledge of their natural way of life in order to safeguard the species. Important areas to understand include the lions' natural corridors of dispersal, areas of genetic interruptions, gene flow and disease patterns (Dubach et al., 2013). Conservation of endangered wildlife species also requires prior insight to their evolutionary processes affecting them. This depends on the knowledge of their historical phylogeographic partitions (Mace et al., 2003; Barnett et al., 2006b).

Conservation of lion populations plays a key role in balancing the ecosystem of the ecological

community by being the apex predator (Ripple et al., 2014), high economic value for ecotourism and trophy hunting (Lindsey et al., 2007a,b, 2012b), cultural values to some communities in Africa and, in other countries, serving as symbols of royalty, totems and sport emblems as well as offering value in some places through legal and illegal trade with their body parts (Williams et al., 2015).

2.3 Threats to African lion

Human dominated and fragmented landscapes have negatively affected biodiversity and left more than 80% of natural ranges for several mammalian species lost (Ceballos et al., 2015; Tensen et al., 2018). Africa has lost most of its biodiversity especially lions to human-borne anthropogenic activities among other threats (Hunter et al., 2007; Riggio et al., 2013; Dubach et al., 2013). Such threats range from loss and fragmentation of habitat, depletion of natural prey base (Becker et al., 2013; Dubach et al., 2013), human-wildlife conflicts (Patterson et al.,2004; Packer et al.,2006; Bauer et al.,2016), illegal trade with bones and other parts of body from lions for traditional medicine (IUCN, 2006a; 2006b; Riggio et al.,2013; Bauer et al.,2015), and poorly managed trophy hunting (Packer et al., 2009, 2011; Croes et al., 2011; Rosenblatt et al., 2014). Others include inbreeding, diseases, climate change and desertification, governance and poverty (IUCN, 2018).

2.3.1 Loss and fragmentation of natural habitats

Anthropogenic activities like agricultural development, infrastructure development and industrialization results to destruction (Frank, 2011) and loss of natural habitat (Loveridge et al., 2007) a home to wildlife species. Natural populations fragment and get isolated from each other as a result of these processes (Bauer et al., 2015). The change of land use in Kenya due to rapid growth of human population lead to dramatic loss of wildlife habitats. This has further

resulted to the decline of African lions witnessed in Kenya and the African continent (Watts, 2016). Because of their large geographic ranges, lions with a history of rapid decline and small population sizes in Kenya has suffered isolation of natural populations and restricted movements (Frank, 2011; Tende et al., 2014 a, b). The small isolated populations with restricted gene flow may experience genetic drifts, population bottlenecks or founder events and fixation of weak alleles due to lowered fitness (Frank, 2011; Woodroffe, 2015). This leads to reduced population sizes characterized by high rate of inbreeding and inbreeding depression (Trinkel et al., 2013).

2.3.2 Lion prey base depletion

The density of lion population depend on the available prey species biomass, normally the large herbivores (Hayward et al., 2007; Bauer et al., 2016). Unsustainable and commercialized trade on bush meat from prey leads to collapse of the lion prey population across African savannah ranges (Lindsey et al.,2013a; Bauer et al., 2016) and extensive prey base depletion (Craigie et al., 2010; Lindsey et al., 2013a, b; Bauer et al., 2015). The major lion prey species consist of 78 herbivores studied in Africa. According to this study, the prey species population trend has decreased by 24% in Southern Africa, 52% in Eastern Africa and by 85% in West Africa indicating a similar trend for lions in these countries (Craigie et al., 2010; Bauer et al., 2016).

2.3.3 Human-Wildlife conflicts

Most lions live in national parks with permeable boundaries to human beings, domestic livestock and wild animals (Blackburn et al.,2016; Hazzah et al., 2017). Changes in land use lead to livestock depredation, human wildlife conflicts, retaliatory attacks, cultural killing, human persecution, social, emotional and political influences (van Eeden et al., 2018; Ontiri et

al., 2019). This is normally prominent among the indigenous Maasai communities and plays a major role in range collapse and lion population decline (Frank et al., 2007; Kissui et al., 2010; Blackburn et al., 2016). Declining natural prey base that resulted to increased human-carnivore conflicts remains a big concern (Mesochina et al., 2010; Winterbach et al., 2013). It has been shown that shift to agro-pastoralism has caused increased human-wildlife conflicts, which in turn causes retaliation attacks on wildlife by the community (Frank et al., 2006). Corruption, mis-management and denying the local communities resources and revenue from wildlife have also contributed to the conflicts of wildlife with local communities (Muriuki et al., 2017).

Approximately 14% of livestock in Africa is lost to wildlife killing. This is estimated to cost US$ 959,240.20 out of which 2.4% is from South Eastern Kenya (Blackburn et al., 2016; Muriuki et al., 2017). It is also estimated that a single lion at Tsavo NP costs Kenyan ranchers approximately US$ 290 per year with regard to loss of livestock (Patterson et al., 2004; Bauer et al., 2016; Muriuki et al., 2017). In 1996, livestock loss to lions did cost Laikipia ranchers approximately US$ (300- 400) (Packer, 2005; Frank et al., 2005). This was frequently experienced during the rainy seasons (Frank, 2011; Parks et al., 2016). About 2.2% of livestock in commercial Ranches next to Tsavo ecosystem and 0.1% of livestock in Mbirikani group Ranch were killed by lions, which accounted for 93% loss of livestock (Patterson et al., 2004; Muriuki et al., 2017). In other areas like the Northern Kenya, lions accounted for 63% livestock loss. In Taita and Rukinga Island in Kenya, lions accounted for 83.5 to 95.7% livestock losses while in Mbirikani group Ranch and in areas surrounding Maasai Mara the lions accounted for 7% losses and 19% livestock losses respectively. There have been reports of lion killing as early as 1998 when the Maasai morans were reported to have speared 87

lions in Nairobi National Park and Kitengela plains. Another 76 lions from Mbirikani group ranch, south east Kenya between Amboseli and Tsavo National Park in 2001 were killed (Packer, 2005; Frank, 2011). At least another 160 lions were poisoned or speared in Mbirikani group ranch (1229 km^2), Olgulului group ranch (1470 km^2) and Eselenkei group ranch (748 km^2) in Amboseli-Tsavo ecosystem between 2003 and 2011 (Hazzah et al., 2014; Hazzah et al., 2017).

However, in other countries like Zimbabwe, in Gokwe Community lands, lions accounted for 5% of livestock losses (Arif et al., 2011; Muriuki et al., 2017). In Cameroon, 3.1% of all losses of livestock representing 22% financial losses which were recorded annually and amounted to US$ 370 for every rancher (Bauer et al., 2016). In the USA, about US$1.65 million losses of sheep and goats have been recorded (Arif et al., 2011; Muriuki et al., 2017). In South-Eastern Tanzania with very low density of lion prey, man-eating is rampant, which in turn causes retaliatory attacks on lions (Packer, 2005; Kushnir, 2009). The killing of 125 lions from Tarangire-Manyara ecosystem and another 35 lions from Ngorongoro conservation area between 1994 and 2004 could be the result of this. In other African countries like Mozambique, human-wildlife conflicts are the major source of lion and livestock mortality. Similar trends were also witnessed in Ethiopia (Packer, 2005; Anderson and Pariela, 2005) which is known as a mammalian diversity hotspot (Antunes et al., 2008; Gebresenbet, et al., 2009, 2018a, b).

2.3.4 Trophy/Sport hunting

Sport or trophy hunting also called “safari hunting” practiced in several countries in sub-Saharan Africa play a crucial role for conservation and management of land set for wildlife (Packer et al., 2006, 2013; Bauer et al., 2016). It provides financial support to both the local

communities and the government (IUCN, 2018). When properly managed and regulated, it is a good conservation tool but turns out to be a threat when poorly managed (Loveridge et al., 2007; Packer et al., 2011). It earns revenue to countries with a rich biodiversity (Loveridge et al.,2007) and offers a reason for conserving available habitats and species (Packer, 2005; Bauer et al., 2016). Corruption and institutional mismanagement remains conservation threats with negative effects to the species population (IUCN, 2018).

Lion populations in stable habitats can hold a given amount of trophy hunts as long as the laid down rules and procedures are strictly followed (Whitman et al., 2004, IUCN, 2018). The population size and structure in fragmented populations are severely affected by uncontrolled sport hunting of wildlife (Brashares et al., 2011; Packer et al.,2011). Hunted male lions are normally replaced by new young males from neighboring protected areas and this affects their population structure (Loveridge et al.,2007). High pressure from both excessive sport harvests and illegal poaching lead to an extra cause of lion decline (Whitman et al., 2004; Frank et al., 2005; Packer et al., 2011).

Although one male lion older than 5 years per 2000 km^2 is recommended for sport hunting of lions (Packer et al., 2011; Tensen et al., 2018), several countries still practice higher levels which is a potential threat (Lindsey et al., 2007a, 2013a,b; Bauer et al., 2016). However, hunting 6 year male lions in Serengeti NP (Whitman et al., 2004; Packer et al., 2011) and 10% adult males in Selous Game Reserve was sustainable (Crosmary et al., 2018). It was estimated to have caused huge population declines in countries like Tanzania, Botswana, Zimbabwe, Namibia (Packer et al., 2009; Packer et al., 2011), Zambia (Rosenblatt et al., 2014; Bauer et al., 2016) and Cameroon (Croes et al., 2011; Bauer et al., 2016). In Zimbabwe's privately owned land, good practice has been seen where some of the funds

generated through trophy hunting has been used to support conservation during unstable political and economic times (Williams et al., 2016; Tensen et al., 2018). These were put in place to ensure good trophy hunting practices to avoid cases like ones seen in Hwange National Park where misuse of funds was experienced (Tensen et al., 2018). Properly regulated trophy hunting contributes positively to management conservation of lions. It is due to this that improvement on conservation strategies has been recommended (Lindsey et al., 2013b; Edwards et al.,2014).

2.3.5 Illegal trade on Lion body parts

Demand for traditional African Chinese medicines poses a great threat to African lion conservation and the small subpopulation in India (Williams et al., 2015). It promotes illegal trade in lion body parts (IUCN, 2018). The regional lion conservation strategies call on countries to prohibit and control trade on lion bones, body parts and products (IUCN, 2006b; Bauer et al., 2016). In other African countries like West and Central Africa, the use of skin from lion, bones and fat have been reported (Faso, 2014; Williams et al., 2017).

More than 22 lion body parts suspected to have been targeted for medicinal use were recovered in Yankari Game Reserve in Nigeria (Born Free Foundation, 2008; Bauer et al., 2016). The use of African lion bones in Asia has paved way for captive lion bones to be used to make medicinal wines together with tiger bones. Exports of authorized bones of captive lion from South Africa to Vietnam and China could encourage the illegal trade on wild lion body parts (Nowell and Pervushina, 2013; Bauer et al., 2016). These requires an urgent protection for the Eastern and Southern African wild lion body parts which could be drawn in to the Asian illegal trade (Bauer et al., 2016).

2.3.6 Inbreeding

Inbreeding is a genetic risk of concern that involves mating of closely related individuals. It has detrimental effects in lowering the survival fitness of affected animals as compared to their ancestral parents. Inbreeding brings about the inbreeding depression on inbred offsprings with characteristic loss of fitness compared to randomly mated or out-bred offsprings. Endangered wildlife species have population sizes that experience inbreeding depression and genetic drift due to fixation of their allele variants that survive bottleneck or founder events (Tensen et al., 2018; Lacey et al., 2019). Genetic drift may at times cause disappearance of fixed alleles with an end effect of loss of genetic variation (Hedrick & Kalinowski, 2000).

The current loss of genetic variation due to range collapse and small population sizes resulting from natural habitat destruction, habitat fragmentation, isolation of natural populations and restricted movements is of big concern (Callens et al., 2011; Tracy and Georget, 2019). In isolated populations, gene frequencies are greatly influenced by two evolutionary forces including the genetic drift and the founder effect (Tensen et al., 2018; Lacey et al., 2019).

Severe bottleneck events, and genetic erosion within lion conservation units like the Ngorongoro crater and Hluhluwe-Umfolozi caused the populations to lose their genetic variability (Dubach et al., 2013; Tensen et al., 2018). More than 200 Save Valley Conservancy lions and 500 to 550 Bubye Valley Conservancy lions of Zimbabwe could also have suffered a similar experience (Tensen et al., 2018). The Zambia lion population also suffered similar fate although the inbreeding coefficient was suspected to have been caused by Wahlund effect (Tensen et al., 2018). Although the lowveld region lions in Zimbabwe experienced high genetic inbreeding and differentiation, the African wild dogs living together with them did not show population genetic differentiation (Tensen et al., 2016; Tensen et al., 2018). The reduced

genetic variation which may have increased extinction risk may have been caused by trophy hunting of males (Lim, 2016; Tensen et al., 2018).

2.3.7 Diseases

African lions are prone to several infectious diseases ranging from viral, bacterial to parasitic. The viral infections include the Feline Herpes virus subtype 1 (FHV-1), Feline Immunodeficiency Virus (FIV_{Ple}), Canine Distemper Virus (CDV), Feline Parvovirus (FPV) and Feline Corona Virus (FCoV) (IFAW, 2011; ALERT, 2011; Trinkel et al.,2013). The Feline Herpes virus subtype 1 (FHV-1) usually infects free-ranging felids and is prevalent in Africa, Asia, Europe and North America (Dybas, 2009; IFAW, 2011; ALERT, 2011). The prevalence of the disease in Etosha and Kruger NPs has been shown to be 67 and 97% respectively while a prevalence of 99 to 100% was recorded in Ngorongoro crater, Serengeti, Central Kalahari region and Lake Manyara region NPs (IFAW, 2011; ALERT, 2011; Roelke et al., 2013).

There are six subtypes of Feline Immunodeficiency Virus (FIV_{Ple}) identified and include subtype A to F. The virus manifests as AIDS-like disease in infected lions with CD4+ depletion, reduced CD4+/CD8+ subtype ratios, immune suppression and death (McEwan et al., 2008; Roelke et al., 2013; ALERT, 2011). Different countries show different prevalence in different subtypes. For instance, FIV_{Ple} subtype E was prevalent in Botswana's Okavango delta lion populations. Among the 93% of the Serengeti lion populations infected with the virus, the lions infected with subtypes A, B and C of the virus accounted for 43% (Troyer et al., 2008; ALERT, 2011).

The Canine Distemper Virus (CDV) was globally distributed in both captive and free-ranging carnivore populations including felids with large scale epidemics. The two major epidemics in

1994 and 2001 that infected 85% of the Serengeti-Mara ecosystem lions killed approximately 1000 lions and were suspected to have been spread by domestic dogs. The Ngorongoro National Park lion population suffered 35% mortality rate during the 2001 epidemic and the presence of the virus in other carnivore species including domestic dogs, spotted hyenas and bat-eared foxes was associated with interspecies infection (ALERT, 2011; O'Brien et al., 2012).

The Feline Parvovirus (FPV), also called Feline Panleukopenia or Feline infectious enteritis (FIE) affects both exotic and domestic felines. The viral prevalence varies in different lion conservation units. The Kruger National Park lion population was suggested to have the highest prevalence rate of 84%, Serengeti (75%), Lake Manyara region (60%), Ngorongoro Crater National Park (27%). The viral outbreaks of Feline Calicivirus (FCV) have been reported in different African countries. Higher prevalence rates were seen in Serengeti plains and low prevalence rates were reported in Botswana while there was no evidence of the virus in Ngorongoro, Tanzania. Feline Corona Virus (FCoV) infects both cats and dogs and develops into Feline Infectious Peritonitis (FIP), a more pathological disease. Varying levels of the virus have been found in the African lion population (ALERT, 2011).

The evidence of bacterial diseases has also been reported. The spread of *Mycobacterium bovis* which caused bovine tuberculosis (bTB) disease in the Kruger NP (southern part) in South Africa was introduced by the domestic cattle. It later spread to the north by African Buffalo. The disease prevalence was 4% in Serengeti NP and was also confirmed in reserves including MHuluhuluwe Umfolozi. Common causes of parasitic diseases in lions include Theileria, Hepatozoon and Babesia. Few cases have been reported in Serengeti and Ngorongoro Crater National Parks in Tanzania. Endo-parasites occurs both as inter-cellular and intra-cellular

organisms in free-ranging lions of Southern Africa. The evidence of the parasite was reported in Congo, Nigeria, Transvaal, Northern Transvaal and Zambia. Many cases were also reported from Zoos in India (Antunes et al., 2008; ALERT, 2011; Adams et al., 2012; Roelke et al., 2013).

2.3.8 Climate change and desertification

Sub-Saharan Africa is experiencing high levels of carbon dioxide resulting to dry hot environmental conditions characterized by high droughts. This is expected to cause an increase of temperature by 6°C in 100 years to come (IUCN, 2018). This would result to large scale migration of wildlife mammalian species resulting into huge loss of wildlife ranges (Thuiller et al, 2006). The African Sub-Saharan also faces desertification due to cumulative effects of climate change, forest loss for timber and charcoal and overgrazing (United Nations Economic Commission for Africa (UNECA), 2007). This could place huge pressure on Africa food production and this will further put a lot of pressure on habitats meant for wildlife conservation (Zewdie, 2014).

2.3.9 Bad governance

Corruption due to political influence is a dreaded threat to wildlife conservation in Sub-Saharan Africa (WWE and Traffic, 2015); because it condones the practice of illegal business in wildlife products (Garnett et al. 2011; WWE and Traffic, 2015; Packer and Polasky, 2018). African countries estimate that about USD 150 billion per year which includes USD 30 billion dollars in form of aid, 25 % Gross Domestic Products (GDP) and 50 % of the continent's tax revenue is lost to corruption through ignorance of the rule of law (UNECA, 2016; IUCN, 2018).

In African countries, corruption is prominent at all levels of government institutions from

lower levels through higher levels to the executives, legislature and judiciary (UNECA, 2016). This has an end effect of poorly managed conservation doomed to fail (WWE and Traffic 2015; IUCN, 2018). This is because this chain of corruption ends up misappropriating funds supposed to be used for conservation (IUCN, 2018). This in turn puts pressure to the relevant institutions and forces them to offer compromised services and reduced tax revenue furthering poverty margins. Illegal activities such as trafficking of wildlife products (Garnett et al. 2011; Wyatt and Cao, 2015; WWE and Traffic, 2015) and poaching find their way at this point of irresponsible governance and misuse of rule of law (WWE and Traffic, 2015; Packer and Polasky, 2018; Baghai et al. 2018).

2.3.10 Poverty

Many countries in Sub-Saharan Africa lie below the poverty margins with their wildlife suffering underfunding (IUCN, 2018). This puts pressure in the relevant conservation institutions in management of their wildlife protection areas (Lindsey et al., 2017a; Packer and Polasky, 2018). Biodiversity protection in Africa is poorly funded in about 33 % of its countries (Waldron et al. 2013). Unlike in North American and European National Parks where conservation of their wildlife is dependent on the countries tax base, African wildlife conservation is dependent on international funding or their GDP (Packer and Polasky, 2018).This is further worsened by Africa's poor technical and scientific capacity in wildlife management (Lindsey et al., 2017a).

2.4 Historical Geographic Distribution of lions

The presence of fossil evidence in Eastern Africa was a suggestion that East Africa could be the origin of lions (Barnett et al., 2006b). The first lion-like cat was found between 5 million and 1.8 million years ago during the late Pliocene in East Africa (Werdelin and Lewis,2005;

Barnett et al. 2006b; Antunes et al.2008). Between 800,000 and 100,000 years ago during the middle Pleistocene period, lions migrated out of Africa into Europe and Asia colonizing the whole of Holarctic region in a similar pattern to that of humans (Yamaguchi et al.2004; Barnett et al.2006b). Lions settled in Europe around 500,000 years and became the most widely distributed among large mammals between 130,000 and 10,000 years ago in the late Pleistocene period. Lions became globally distributed (Barnett et al., 2006b; Tuqa et al., 2014) with their home ranges in Africa, Asia, Europe and America (Barnett et al.,2009; Riggio et al.,2013).

The human population pressure has increased anthropogenic developments in areas which were formally lion ranges. This has led to range reduction, fragmentation of habitats and lion populations (Smitz et al.2018). An alarming reduction of lions has resulted from these changes (Chardonnet et al. 2010; Riggio et al. 2013; Lindsey et al. 2013). Other factors such as the unstable climatic changes during the Pleistocene period also led to the species range collapse over much of the northern and western Eurasia and into North America. The collapse of the several species in the Holarctic regions due to increased human population growth caused serious disruption over the species range (Ersmark et al., 2015). This led to the extinction of the south-west Asian lion population leaving the critically endangered Gir forest lions, the cape and the Barbary lions (Dubach et al.2005). This similar trend of lion extinction was seen in most countries like Greece, Palestine, Algeria, Turkey, Tunisia, Iraq, Iran, Morocco, southern part of South Africa between 1870 and 2000 (Patterson et al., 2004; Ersmark et al., 2015).

2.4.1 Geographic Distribution of lions in Africa

Although there are several studies done on lions among the large felids, rare information is

available on their population estimates in Africa. This has been due to inadequate resources, unfavorable policies, political and economic instability as well as institutional inadequacies on states wildlife management authorities (Packer et al., 2013; European Commission, 2015; McKinnon et al., 2016). Limited allocation of funds and difficulty in counting lions has also been a major hindrance in lion survey (Bauer and Van Der Merwe, 2004).

In 1911, before European colonization, lion estimate in Africa was about 1.0 million lions which declined steadily after colonial rule (1950) to about 500,000 lions. By 1975, lions declined further to 200,000 and to less than 100,000 by early 1990 (IUCN, 2006 b; Riggio and Pimm, 2011). It is estimated that for the past 21 years, the African lion population have declined by at least 43% (Bauer et al., 2015; Hazzah et al., 2017) (Figures 2.1). The IUCN has estimated that between 20,000 and 39,000 lions live in African continent distributed across 27 countries at an estimated area of 3 million km^2 and 78 habitat patches. About 60% of the lion population in Africa are found in East Africa (IUCN, 2006 b; Hazzah et al., 2017) of which half of this population resides in Tanzania (Mesochina et al., 2010). In Africa, seven countries, three in Eastern Africa (Kenya, Tanzania and Ethiopia) and four in Southern Africa (South Africa, Botswana, Namibia and Zambia) have up to 1000 or more lions (IUCN, 2006 b; Riggio and Pimm, 2011).

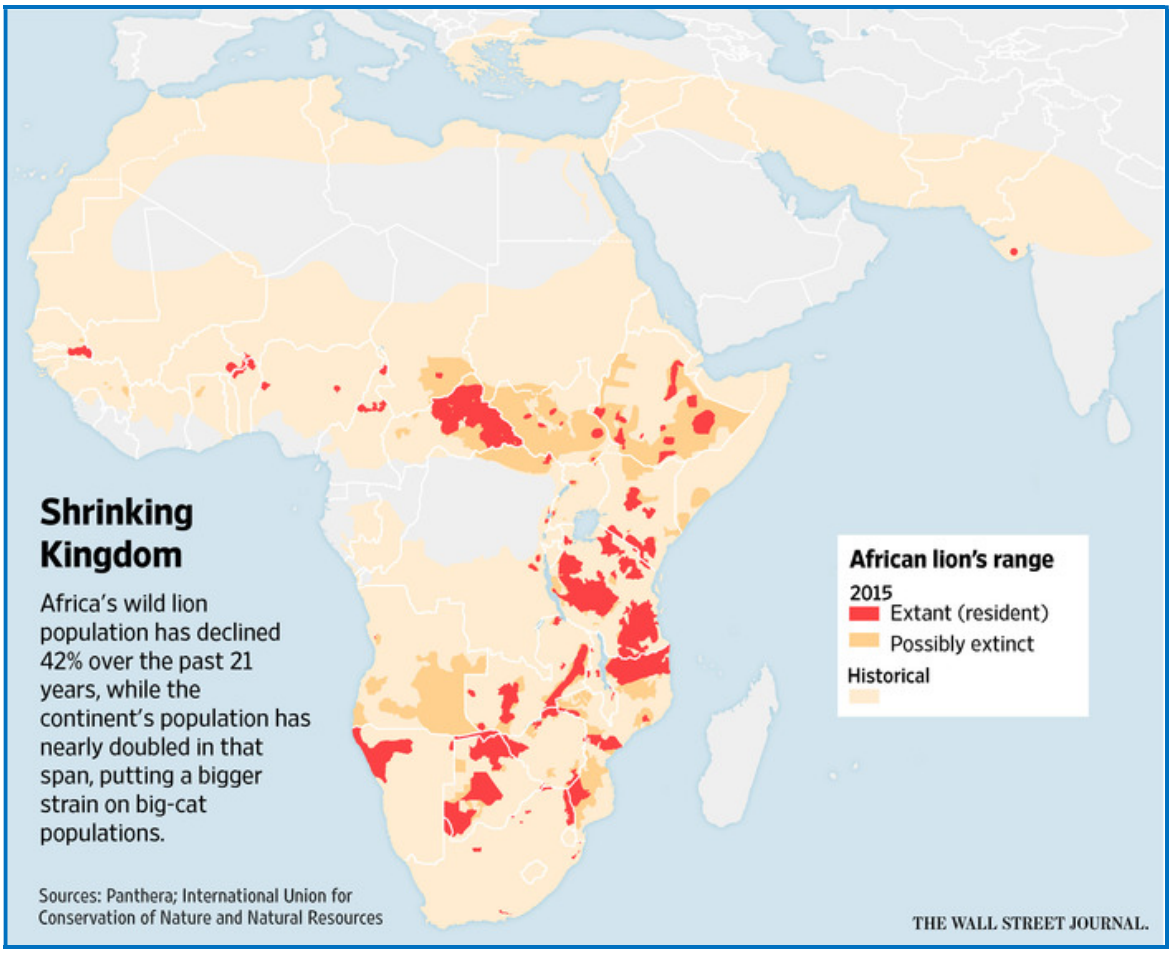


Figure 2.1: Map of Africa showing the African lion range, areas with Red indicates possible areas with living lions, areas with brown indicates area possibly where lions are extinct. Source:(Bauer et al., 2015)

2.4.2 The Eastern and Southern Africa lion (*Panthera leo melanochaita*) populations

Panthera leo melanochaita lion populations are found in Eastern and Southern Africa. In Eastern Africa, the population could be extinct in Egypt, Eritrea and Djibouti (IUCN, 2006b) and occurs in Kenya, Somalia, Ethiopia, Southern Sudan, Uganda and Tanzania (Bauer et al., 2016). The Southern African lion populations also known as Cape lions occurs in South Africa, Malawi, Botswana, Swaziland, Zambia, Zimbabwe and Mozambique (Figures 2.2). These lion populations in Southern Africa are genetically close to those in Western Africa, Northern Africa and Asia (Riggio and Pimm, 2011; Bertola et al., 2011). Phylogenetic studies suggested that, the *Panthera leo melanochaita* lion population occur as admixture of lion populations from different parts of Eastern Africa in some regions including North Western Kenya, Western Somalia and South Eastern Ethiopia (Riggio and Pimm, 2011).

It was estimated that East Africa has four lion strong-holds (Riggio and Pimm, 2011). These include the Serengeti-Mara ecosystem, Tsavo-Mkomazi ecosystem, Ruaha-Rungwa ecosystem and the Serous Game Reserve. The Serengeti-Mara ecosystem transverses the Tanzania and Kenyan borders in an estimated area of 57,800 Km^2 with census reports suggesting varied estimates of lion populations (IUCN, 2006b; Riggio and Pimm, 2011). A total lion population of between 3,131 and 5,378 lions was estimated to live in this ecosystem. Between 825 and 941 lions were suggested to live in the Kenyan side and between 2,848 and 3,896 lions in the Tanzanian side (IUCN, 2006b; KWS, 2009; Mesochina et al., 2010). The MMNR which lies in south-western Kenya holds about 540 lions within its 1,800 Km^2. It had the highest population density of 30 lions per 100 Km^2 (Riggio and Pimm, 2011).

The Tsavo-Mkomazi ecosystem along the border of Kenya and Tanzania had an estimated

population of between 675 and 880 lions (IUCN, 2006b; KWS, 2009). It was estimated that approximately 205 lions lived in the Tanzanian side and the majority of the population lived in protected areas (Mesochina et al., 2010). The Ruaha-Rungwa ecosystem in Western/Central Tanzania with approximately 185,540 Km^2 had an estimate of 3,779 lions. The Serous Game Reserve ecosystem in Southern Tanzania covers approximately 190,380 Km^2 with an estimate lion density of 8 lions per 100 Km^2 (Riggio and Pimm, 2011). It was estimated that the ecosystem has 7,268 lions of which 4,353 lions are in its protected areas (IUCN, 2006b; Riggio and Pimm, 2011).

About six lion strongholds are estimated to be found in Southern African lion populations. These include the Niassa ecosystem, Luangwa ecosystem, Mid-Zambezi ecosystem, Kgalagadi ecosystem, Ol-Kavango-Hwange ecosystem and Greater Limpopo ecosystem. The Niassa ecosystem in Northern Mozambique has two LCUs, the Greater Niassa and the Niassa Reserve with an area of 128,000 Km^2 (IUCN, 2006 b; Riggio and Pimm, 2011). Approximately between 175 and 1,949 lions live here with 1,080 lions in protected areas (Bauer and Van Der Merwe, 2004; Mesochina et al., 2010). Out of this estimate, between 800 and 900 lions live in the Niassa Reserve alone and between 100 and 250 lions live in the Greater Niassa ecosystem. The Luangwa ecosystem has several national parks and game management areas in Eastern Zambia. It stretches between Luangwa River valley in Zambia and northern Malawi. An estimate of between 425 and 850 lions live in a 77,041 Km^2 conservation area.

The Mid-Zambezi ecosystem lies between South-Eastern Zambezi and Northern Zimbabwe along the Zambezi River and Kariba Lake. Its 42,142 Km^2 conservation area had an estimate

of between 350 and 988 lion population within its national parks and wild management areas (IUCN, 2006 b; Riggio and Pimm, 2011). The Ol-Kavango-Hwange ecosystem covers approximately 95,170 Km^2 between Northern Botswana and Zimbabwe. An estimate of between 2,118 and 2,870 lions live within its 107,337 Km^2 conservation area (Bauer and Van Der Merwe, 2004; IUCN, 2006 b). The area was also estimated to hold a population density of 2.7 lions per 100 Km^2 in 14,900 Km^2 area around Hwange National Park alone with 402 lions (Loveridge et al., 2007; Riggio and Pimm, 2011). The Kgalagadi ecosystem transverses a vast area between Central and northern South Africa. A population of between 500 and 1,150 lions in an area of 151,142 Km^2 was estimated in this ecosystem. The Greater Limpopo ecosystem approximately 60,990 Km^2 with an estimate of between 2,000 and 2,793 lions transverses several national parks and national game areas in South Africa, Mozambique and Zimbabwe. Approximately 2,000 lions were found in Southern Africa with more than 1,957 lions thought to be in well protected areas (IUCN, 2006 b; Riggio and Pimm, 2011). Out of this, 1,684 lions lived in Kruger National Park alone (Ferreira and Funston, 2010) while 376 lions lived in Mozambique (Chardonnet et al., 2010). According to IUCN, all the lion populations in all these strongholds were stable (Riggio and Pimm, 2011).

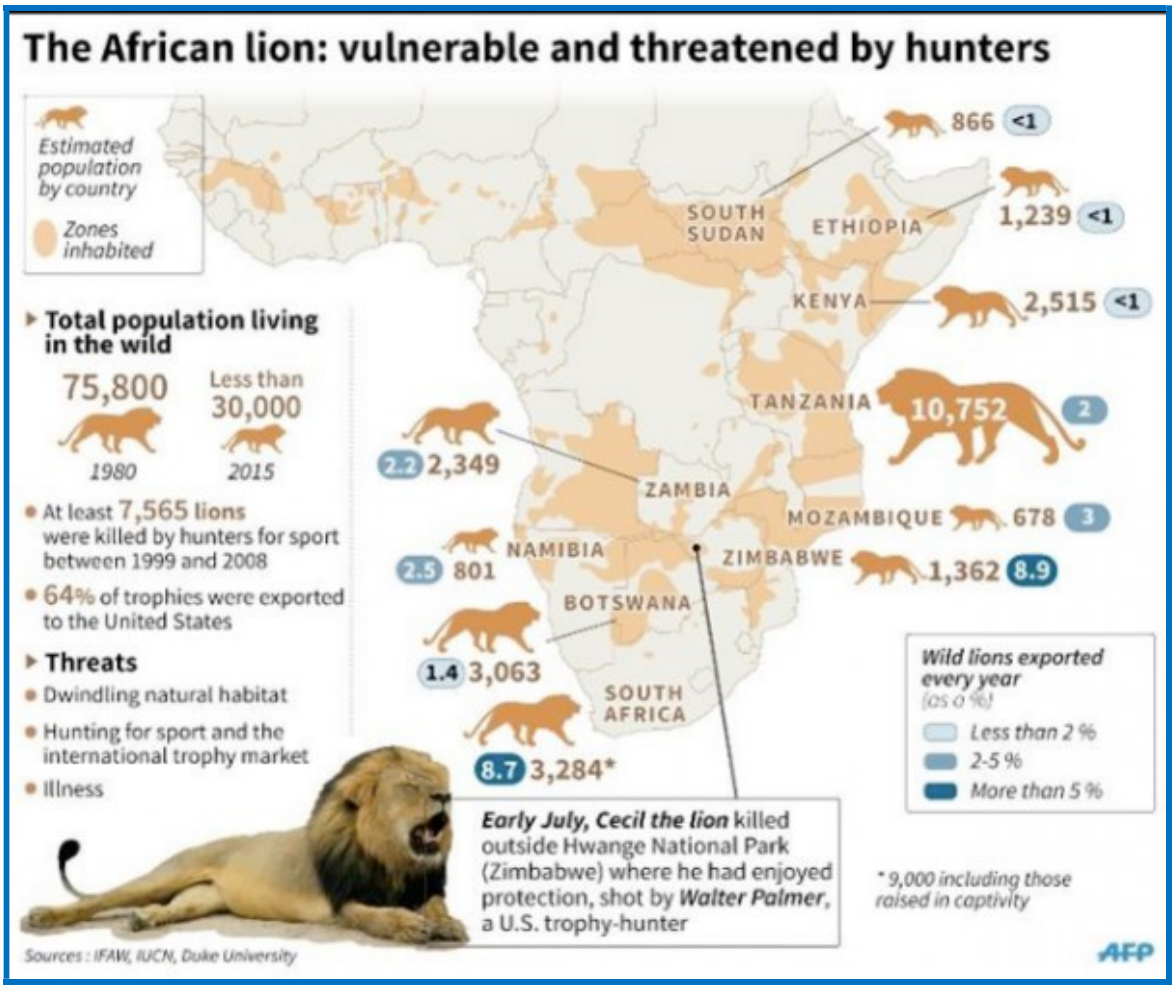


Figure 2.2: Population estimate of lion in Africa by numbers, Figures indicated corresponds to lion estimates per country by 2015. Note the lion population in Kenya was 2,515<1 by 2015. Source: (Lion Aid, 2015).

2.4.3 Lion Population in Kenya

It was approximated that Kenya has 19% of its total land mass as wildlife protected areas suitable for lion habitats. Kenya was estimated to have 2,749 lions by 2002 survey (Muriuki et al., 2017). In 2013, the KWS estimated that about 2,000 lion populations lived in Kenya. Earlier estimates approximated between 1,970 and 2,000 lions with 825 lions living in the Maasai land (Frank et al., 2006; KWS, 2008; 2009; Muriuki et al., 2017). Lions were known

to cover half of the country in the northern Kenya, southern Kenya and most of the Maasai land though there has been several changes in the past twenty years (Frank et al., 2005). The dramatic decline was ascribed to spearing and poisoning due to lion human conflicts among others (Blackburn et al., 2016). This has been prominent in the Tsavo-Amboseli ecosystem and northern Laikipia with huge losses of lions being experienced (Frank et al., 2006; Frank, 2011).

Kenya has two Lion Conservation Units that form two among the four major East Africa's lion strongholds (Riggio et al., 2013). The Greater Maasai Mara Ecosystem in south western Kenya forms the Greater Mara-Serengeti ecosystem between Kenya and Tanzania (Riggio and Pimm, 2011). According to IUCN, approximately 3,500 lions live in the entire Mara-Serengeti ecosystem. The Maasai Mara ecosystem has an estimate of between 558 and 941 lions (IUCN, 2006 b; KWS, 2009; Riggio and Pimm, 2011). The Tsavo National Park forms part of the bigger Tsavo Mkomazi ecosystem that transverses south western Kenya and Tanzania (Riggio and Pimm, 2011). The ecosystem was estimated to contain 675 and 880 lions (IUCN, 2006 b; KWS, 2009; Mesochina et al., 2010). Other Lion Conservation Units outside these strongholds in Kenya include the Maasai Steppe covering the Nairobi National Park with an estimate of 22 lions, Amboseli National Park with an estimate of 60 lions and a density of 0.03 lions/km^2 between 1999 and 2002 (Okello, 2005; 2010; Muriuki et al., 2017), Meru National Park, Kora National Park and several other National Parks in Central Kenya with an estimate of between 40 and 250 lions (IUCN, 2006 b; Riggio and Pimm, 2011; Frank, 2011). Laikipia being the only known non-protected lion habitat with a stable lion population has an estimate of 200 to 300 lions in its commercial ranches and a density of 6 to 7 lions/100 km^2.

The lion pride size density in Kenya's Tsavo National Park ecosystem was estimated at 4 adult

female lions/100 km^2 (Patterson et al., 2004; Frank, 2011) while the Mbirikani group ranch in Maasai land had a population density of 1.5 lions /100km^2 (UCN, 2006 b; Frank et al., 2006; Maclennan et al., 2009). In other areas like the north of the Maasai Mara National Reserve, lion population density was estimated at 12% (Packer, 2005; Ogutu et al., 2005). Compared to other conservation units in other countries like South Africa, the Kruger National Park had a lion population density of between 3.3 and 9.6 adult and sub adult lions per100 Km^2 respectively. The Selous Game Reserve had an estimate of between 8 and 13 adult and sub adult lions/100 Km^2 (IUCN, 2006 b; Riggio and Pimm, 2011). The lion population density in Zambia, on the other hand, was estimated at approximately 5 adult and 6 sub adult pride females per100 Km^2 and a density of 12 to 13 lions per100 Km^2 for all its pride sizes (Frank, 2011).

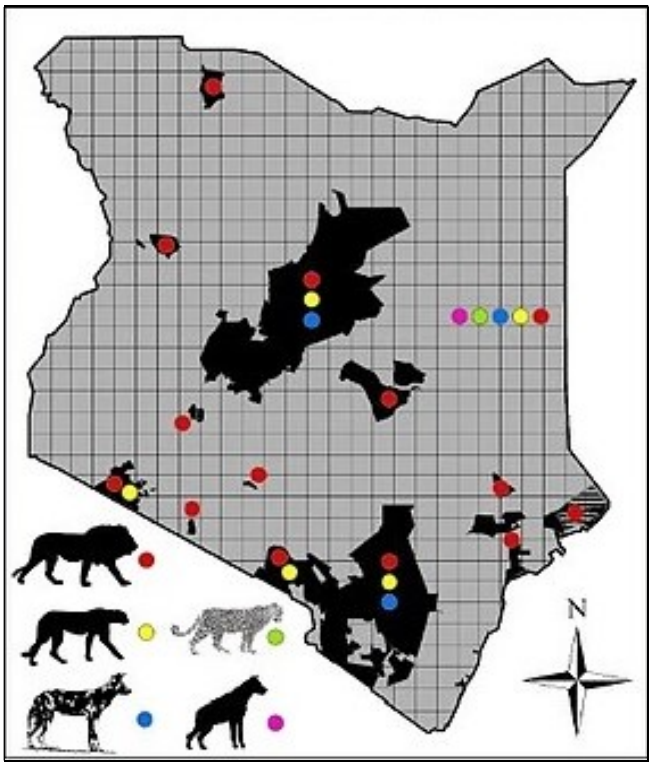


Figure 2.3: Kenya's nationwide lion range and predator survey, lion ranges

are indicated by dots with red, predator ranges are shown by yellow dots, source: KWS

2.5 The Phylogeographic patterns in Lions

Phylogeographic patterns and taxonomic groups distributed together provide crucial information on biogeographical patterns and evolutionary histories important to make conservation decisions (Barnett et al.,2009; Charruau et al.,2011; Bertola et al.,2016). The Great Rift Valley, Sahara desert and the dense equatorial rainforest are three major dispersal barriers that impact negatively on lion distribution patterns in Africa (Burger et al.,2004; Dubach et al.,2005; Barnett et al.,2006a,b). The African Savannah mammals show similar phylogenetic patterns of distribution as indicated by the lion conservation strategy for lions in Africa with lions having a north-south distribution axis (Figure 2.4) (Barnett et al.,2009; Charruau et al.,2011; Bertola et al.,2016). The lions in Central and West Africa (*Panthera leo leo*) have also been shown to have a close relationship different from that in Southern and Eastern Africa lions (*Panthera leo melanochaita*) (Figure 2.4) (Lorenzen et al.,2012; Bertola et al.,2016). Other large carnivores and ungulates in Africa displayed similar patterns of distribution (Okello et al., 2008). These include the wilde beest (*Connochaetes taurinus*), giraffe (*Giraffa siceros*), zebra (*Equus zebra*) (Moodley and Harley,2005; Bock et al.,2014), sable antelope (*Hippotragus niger*) (Brown et al.,2007; Bock et al.,2014) and others (Muwanika et al.,2003; Alpers et al.,2004). According to the phylogenetic studies of African lions, their genetic diversity does not match the former taxonomic phylogeny (White et al.,2013; Barnett et al.,2009, 2014; Bertola et al.,2016). This is because the Asiatic lions sub-species share similar phylogenetic clade with Central and West African lions yet classified differently (Bertola et al.,2011; Charruau et al.,2011; Barnett et al.,2014).

Phylogenetic studies of 130 bp mtDNA D-loop region and 200 bp mtDNA cytochrome *b* region defined the distribution of both the modern and extinct lion populations in a north-south

axis with six phylogenetic clades (Figure 2.4). Among these were three populations from the north including North African/Asia, Central and West Africa and three populations from the south including the Eastern-Southern African, North East Africa and South West Africa (Barnett et al., 2006b, 2014; Bertola, et al., 2016). These phylogenetic groups were also an indication of possible important habitual areas during the recent late Pleistocene climatic changes (Hewitt,2004; Lorenzen et al.,2012). This phylogenetic study showed that the Asiatic lions were closely related to the northern range lions with similar cranial morphology which differentiated them from sub-Saharan African lions (Mazak.,2010; Barnett et al.,2014).

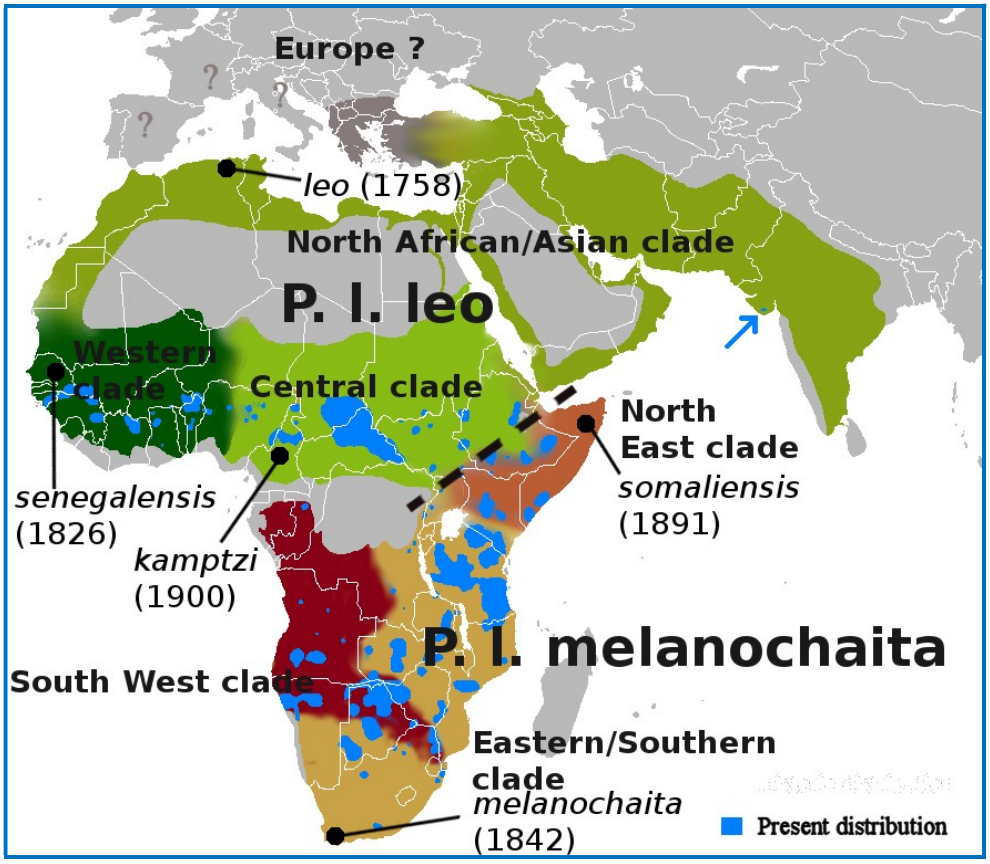


Figure 2.4: Map of Africa showing the six phylogeographic clades of African and Asian lion Source: (Bertola et al., 2016)

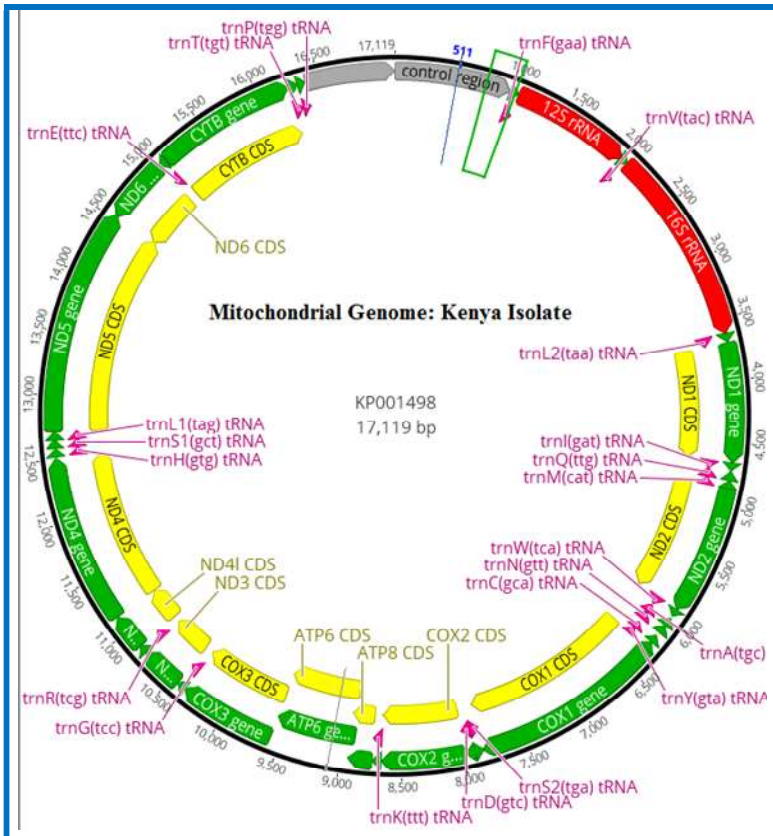
2.6 Mitochondrial genome of the African lion

The mitochondrial genome is found in the organelle mitochondrion which is semi-autonomous, having its own genome. It plays a key role in replication, transcription and protein synthesis in addition to oxidative phosphorylation for generation of energy required by the cell (Jae-Heup et al.,2001; Gupta et al.,2015). The mitochondrial genome encodes 37 genes including 13 protein coding genes, 22 tRNAs, 2 rRNAs (12S rRNA and 16S rRNA) and 1 non- coding control region also called the displacement loop region (D-loop) (Ma et al., 2015) in most mammals including lions (*Panthera leo*) (Cho et al., 2013; Wang, 2014; Gupta et al., 2015; Ma et al., 2015). This is illustrated by the mitochondrial genome of the Kenyan lion isolate (Figure 2.4) below. The mitochondrial genome is a mutation hotspot with high mutation rates due to inefficient DNA repair mechanisms, lack of protective histones and effects of reactive oxygen species (ROS) (Sharma et al., 2019).

The mitochondrial D loop being hyper variable region has essential components for replication and transcription. It also occurs in high number of copies (10^3 to 10^4 copies per cell) in almost all body cells and, most of these copies are usually identical at birth. The heavy (H) and the light (L) complementary strands of mitochondrial genome are based on the guanine (G) quantity. The H-strand is rich in guanine encoding 28 out of the 37 mitochondrial genes whereas the L-strand encodes the remaining 9 genes. Apart from ND6 sub-unit and 8 tRNAs genes, most of the genes in the mitochondrial genome are encoded on the H-strand (Gupta et al., 2015).

The mitogenome of the African lion (*Panthera leo leo*) is a circular and double stranded DNA measuring 17,054bp in length with a Gene Bank accession number KF776494 in the National Center for Biotechnology Information (NCBI) database. The Kenyan lion mitochondrial

genome isolate measures 17,119 bps long with the mitochondrial D loop region measuring 1,668 bps long and has a gene bank accession number KP001498 in the NCBI as shown in figure 2.7 below. The genome size, contents and the number of genes in the genome are similar in all panthera genus with a base composition of A (32.0%), C (26.5%), T (27.0%) and G (14.0%) (Cho et al., 2013; Wang, 2014; Ma et al., 2015). The mitochondrial DNA of felids is unique in that at chromosome F2 in the nuclear, it has a 12.5 kb mitochondrial macrosatellite DNA (numt) segment (Antunes et al., 2008; Jae-Heup et al.,2008). Studies showed that it may translocate and insert itself into any copy of the nuclear within the genome (Jae-Heup et al., 2008).



LEGEND

CYTB: Cytochrome B	**ND 1-5:** NADH Dehydrogenase 1-5
CDS: Coding Sequences	**ATP:** Adenosine Triphosphate
tRNA: Transfer RNA	**RNA:** Ribonucleic Acid
rRNA: Ribosomal RNA	**NADH:** Dihydronicotinamide
COX: Cytochrome Oxidase	Adenine-Dinucleotide Dehydrogenase

Figure 2.5: The mitochondrial genome showing the D loop region of the Kenyan isolate, Green (13 protein coding genes), Yellow (13 Protein coding sequences), Red (12S and 16S rRNAs), Purple (22 tRNAs), Grey (Control region/D loop region), Adapted from Adapted from National Centre for Biotechnology Information (NCBI).

2.6.1 Mitochondrial D-loop

2.6.1.1 The structure

The mt D loop is the longest region that does not encode for any gene in the animal mitogenome. The region contains a third strand of DNA that forms a semi-stable structure

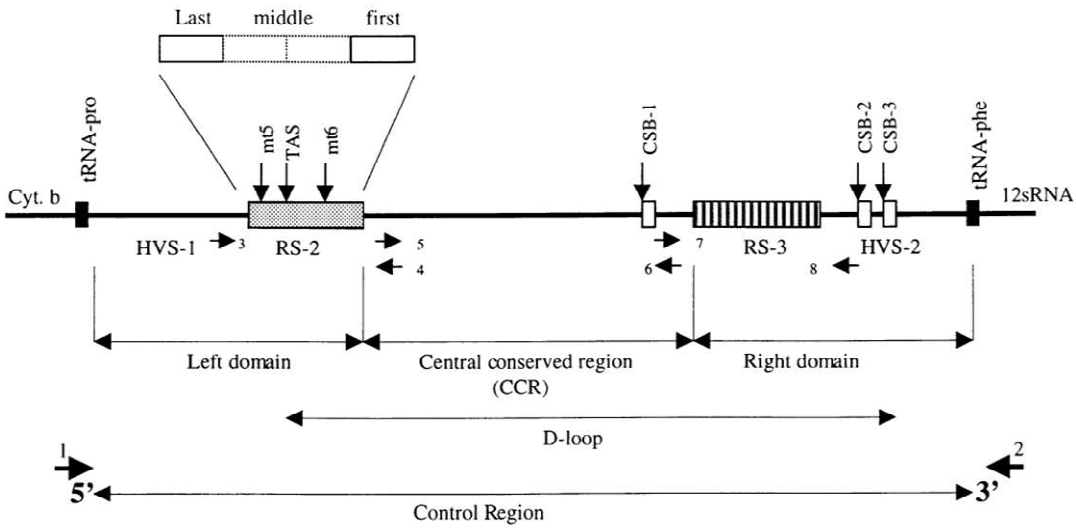
(Bronstein et al., 2018). The D loop is synthesized off the L strand and displaces the mitochondrial DNA H strand. It is initiated near the conserved sequence blocks (CSBs) 1 to 3 and terminates near the Termination Associated Sequences (TAS) (Jae-Heup et al.2001; An et al.,2010). Three main domain regions were identified in five panthera species including the lion (*Panthera leo*), leopard (*Panthera pardus*), jaguar (*Panthera onca*), tiger (*Panthera tigris*) and snow leopard (*Panthera uncia*) (Figure 2.5) (Jae-Heup et al. 2001). They include the; central domain consisting of the central conserved region (CCR) and two flanking A-T rich domains i.e. left domain (L domain) at the 5′ terminal and the right domain (R domain) at the 3′ terminal of the D loop. The three domains are characterized by mutation hotspots or stretches of DNA sequences with several Insertion-Deletion polymorphisms and substitutions. The central domain contains the CCR with conserved sequence block 1 where the Origin of Replication (OR) is located (Jae-Heup et al.2001; An et al.,2010). The CCR located between the RS 2 terminal and the start of the RS 3 ranges between 476 bps to 479 bps in the Panthera genus (Jae-Heup et al.2001). The region has most of its sequences being conserved within individuals and among species with intra individual sequence variations (heteroplasmy) in seven sites. Sequence block D and CSB1 have been identified in this region (Jae-Heup et al.2001). In the L-domain of the D-loop, three structures were apparent including the hypervariable segments I (HVS 1), repetitive sequence 2 (RS 2) and conserved sequence blocks mt5 and mt6. The HVS 1 in Panthera genus range between 188 and 200 bp sequences. They display differences in sequence length due to Insertion-Deletion polymorphisms from tRNA-Pro that starts from 80 bp downstream. The HVS 1 also displays high level of inter-specific sequence variation and similar levels of heteroplasmic sequence sites than HVS-2. This region has both intraspecific and intra-individual sequence variation. The RS-2 flanked

by HVS-1 and located at the sequence block mt5, mt6 and TAS have 80 bp to 320 bp repeat motifs with a range of 1 to 4 repeats. In all the RS-2 repeats, there is a consensus sequence of TAS and mt5 within the first 40 bp (5′ to 3′ ends) while in the first and middle repeats, mt6 sequence blocks are found (Jae-Heup et al. 2001; An et al., 2010). Comparing the 80 bp repeat motifs in RS-2, *Panthera leo* and *Panthera pardus* have maternal close relationship and share a common ancestor with *Panthera onca*. In the R-domain of the D-loop, three structures were also evident, which include the hypervariable segment 2 (HVS-2), repetitive sequence 3 (RS-3) and conserved sequence blocks (CSBs) 2 and 3. The HVS-2 in lion, leopard, snow leopard and tiger ranges between 289 and 298 bps while in jaguar it ranges from 303 to 305 bps. The RS-3 is located between the conserved sequence block 1 and 2, and the arrangement of its specific motifs is highly variable and heteroplasmic (intra-individual variations) (Hoelzel et al., 1987). Although the arrangement of these motifs is species-specific, its basic arrangement is conserved as its molecular signature (An et al., 2010). The RS-3 array has 6 bps to 8 bps repeat motifs with between 28 and 49 repeats and a total of between 238 bps and 376 bps. The RS-3 has a repeat core of TACACG fundamental from which 12 more repeat motifs could be derived through substitutions, duplication and deletion (Jae-Heup et al., 2001).

Most size variation has been attributed to different number of RS-2 and RS-3 repeats in the D-loop region (Jae-Heup et al., 2001). The presence of both repetitive sequences (RS-2 and RS-3) is very rare in all other species but a special case occurs in Felidae family where the two arrays normally co-exist (Jae-Heup et al. 2001; An et al., 2010). The conserved sequence blocks (CSBs) mt5, mt6 on the L-domain (5′ end), the CSB 1 on the central domain and the CSBs 2 and 3 on the R-domain (3′ end) of the D-loop are implicated in H-strand replication. They contain components for initiating replication and transcription of the mitochondrial DNA

by preparing the H-strand for its synthesis (An et al., 2010; Gupta et al., 2015). The termination associated sequences contain the termination signal for replication to terminate the synthesis of H-strand, which occurs during replication.

The African lion mitochondrial D-loop located between $tRNA^{Pro}$ and $tRNa^{Phe}$ in the mitochondrial genome is 1,602 bp long. It‘s between nucleotide positions 16,318 to 17,054 and positions 1 to 865 bp (Cho et al., 2013; Wang, 2014; Ma et al., 2015). On the other hand, the mitochondrial D-loop of the Kenyan isolate measures 1,668 bp long and is located between $tRNA^{Pro}$ and $tRNa^{Phe}$ at nucleotide positions 16,396 to 17,119 and positions 1 to 944 in the mitochondrial genome. Compared to the Asian lions (*Panthera leo persica)*, the mitochondrial D-loop region measures 1,364 bp long and is located between $tRNA^{Pro}$ and $tRNa^{Phe}$ in the mitochondrial genome between nucleotide positions 16,321 and 16,817 and 1 to 867 in the mitochondrial genome (Tabasum et al., 2016).



LEGEND

RS 1/2: Repetitive sequences 1/2	**Cyt b:** Cytochrome b
HVS: Hypervariable segment 1/2	**RNA:** Ribonucleic Acid
TAS: Termination Associated Sequence	**tRNA-Phe:** Transfer RNA-Phenylalanine
CSB: Conserved Sequence Block	**tRNA-Pro:** Transfer RNA-Proline
CCR: Central Conserved Region	**rRNA:** Ribosomal RNA
D-LOOP: Displacement Loop	**12S rRNA:** 12S Ribosomal RNA

Figure 2.6: Showing detailed *Panthera leo* mitochondrial Control Region (D loop).

Source: (Jae-Heup et al. 2001)

2.7 Mitochondrial DNA in conservation

Morphometric analysis and craniometric data have historically been used to identify and establish phylogenetic relationship between lions based on geographical regions (Sotnikova and Nikolskiy, 2006; Mazak, 2010). This information was important for establishing the distinct populations between geographical regions (Yamaguchi et al., 2004; Patterson et al., 2004; Patterson, 2007). However, morphological features did not show true species phylogeny and some of these features were shared (Sotnikova and Nikolskiy, 2006). The patterns of

mitochondrial DNA in lion and other species clearly define the modern African lion phylogenetic and ancestral lineage and avails important information for wildlife conservation (Dubach et al., 2005; Bertola et al., 2016). Management of the species genetic diversity for conservation programmes is vital to protect the genetic resources especially for vulnerable and threatened species (Gupta et al., 2015). The mtDNA has several essential features applicable in animal phylogenetic studies. These include the high mutation rates, maternal inheritance, lack of recombination events, high copy numbers, haploid inheritance and high level of polymorphism (Burton, 2009; Xia, 2013; Gupta et al., 2015; Bronstein et al., 2018). These highly invaluable features play critical roles in evolutionary biology, molecular ecology and population genetics for species conservation and preservation of breeds (Gupta et al., 2015).

The mitochondrial DNA is a molecular clock that defines the species genetic history (Gupta et al., 2015). It also helps in identifying population conservation units, population connectivity and probable dates of population divergence (Endicott et al., 2009; Subramanian et al., 2009; Knaus et al.,2011). For example, the mtDNA cytochrome b and the mtDNA 12S and 16S rRNA calibrated with fossil records from panthera leo estimated 14,000 to 203,000 and 145,000 to 502,000 years before present respectively as the probable time to the most recent common ancestor (TMRC) for modern lions (Antunes et al.,2008; Dubach et al.,2013; Barnett et al.,2014). The mtDNA D-loop region has also been used to define three sub-populations of humpback whales into separate conservation units in Florida, South America. It has been used to offer solutions in understanding evolutionary significant units and taxonomic organization in birds (Kirchman and Franklin, 2007); fishes (Zhao et al., 2006), deer (An et al., 2010), humans (Royrvik et al., 2016) and whales (Felix et al., 2012).

The mitochondrial genome has been used to analyze and characterize biodiversity in animal

species, construct phylogeny of species and determine the genetic diversity and migration routes (Pang et al., 2009; Knaus et al., 2011; Gupta et al., 2015; Bronstein et al., 2018) in various species. In lions, 130 bps mtDNA D-loop region and 200 bps mtDNA cytochrome b region were used to show the phylogeographic distribution of modern and extinct lions in to six phylogenetic clades in a north-south axis. Among these were three populations from the north including North Africa/Asia, Central and West Africa and three populations from the south including the Eastern-Southern African, North East Africa, and South Western Africa (Barnett et al., 2006b, 2014; Bertola, et al., 2016). Also, the mtDNA cytochrome *b* of 75 lions and 11 microsatellite loci in 480 lions from 8 range states of eastern and southern Africa also identified two clades in Eastern and Southern Africa. Among these two clades, one was more wide spread across the regions and the other one was restricted to Namibia and South Africa (Dubach et al., 2013). The mtDNA D-loop and cytochrome b profiles which clustered the Zambian lions to Eastern and Southern African also grouped the Zambian lion population into two groups which included the Southern-Eastern Africa group and Western, Central and North African group (Barnett et al., 2006a, 2014; Curry et al., 2015). According to mtDNA 12S and 16S rRNAs regions, the genetic diversity and matrilineal distribution of the five main lion sub-populations in Zambia comprising the lower Zambezi, Kafue, Sioma Ngwezi, North and South Luangwa between 2004 and 2012 had similar suggestion to a previous study (Curry et al., 2015). The previous study had suggested that the Eastern and Southern African lion population was the evolutionary origin of the lion (Barnett et al., 2006a, 2014; Curry et al., 2015). The study also suggested that Zambia could possibly be the genetic corridor between the Eastern and Southern Africa lion populations (Curry et al., 2015). The mtDNA and feline immune deficiency viral gene was used to show the low patterns of genetic variations among

lions in some lion ranges including Kenya, Southern and Eastern Africa, South Africa, Asia, North Africa and Central Africa (Antunes et al.,2008). In Ethiopia, the mtDNA cytochrome b and 10 microsatellites loci markers were used to define the genetic diversity of 15 captive Addis Ababa lions and to show that they belonged to African lions (Tefera, 2003; Frank, 2011). The genetic variations of 358 bps mtDNA D-loop and ATPase subunit 8 suggested that there were three lion species including the modern lions (African and Asian sub-species), the Cave lions (Asia and North America) and the American lions (southern North America) (Antunes et al., 2008; Bertola et al., 2011; Dubach et al., 2013). The mtDNA has further been used in analysis of genetic distance between species which helps to construct distinct population genetic structures of the studied species (Gupta et al., 2015). The mtDNA cytochrome b and microsatellite loci genetic markers were used in Ethiopia, Addis Ababa to show the genetic similarity and physical closeness of Yankari Game Reserve and Kainji Lake National Park lions with lions from Cameroon and Benin respectively (Tende et al., 2014b). They also showed the high levels of inbreeding with in-breeding coefficient (F_{IS}) of 0.21 in YGR between 2008 and 2012. In a study conducted later between 2009 and 2012 the in-breeding coefficient (F_{IS}) was found to be 0.49 in YGR and 0.38 in KLNP (Tende et al., 2014a). The observed levels of in-breeding and in-breeding coefficient were suggested to be as a result of separation of the small population for many generations with possible loss of genetic variations (Lande, 2003; Tende et al., 2014a; Laikre et al., 2018).

The mtDNA was further used to show high levels of in-breeding and its negative impacts in Tanzania's Ngorongoro National Park lions (Packer et al., 2009; Tende et al., 2014a). This similar trend was also shown in other species including Scandinavian wolf (*Canis lupus*) with an F_{IS} of 0.41 (Tende et al., 2014a; Laikre et al., 2018) and Brown bear (*Ursus arctos*) with an

F_{IS} of 0.37 (Tende et al., 2014a; Akesson et al., 2016). Similar levels of in-breeding were also observed in Namibia's Etosha National Park (F_{IS} = 0.37) (Antunes et al.,2008; Lyke et al., 2013; Tende et al., 2014a). The low levels of gene flow and differentiation among the Coyote (*Canis latrans*), mountain lions (*Puma Concolor*) and bobcat (*Lynx rufus*) in California was also an evidence of the underlying threats. This probably could have been caused by anthropogenic obstacles that included urbanization, roads and agriculture that prevented dispersal and gene flow. They also imposed artificial home ranges and reduced genetic effective movement that led to genetic delineation among these territorial carnivores (Riley et al., 2006; Castilho et al., 2012; Tende et al., 2014a).

Because of its high level of sequence polymorphisms, haploid maternal inheritance and high copy numbers, the mitochondrial D-loop is useful in forensic science, epidemiological studies, solving taxonomic questions and disease diagnosis (Gupta et al., 2015; Xie et al., 2018). It is an essential marker for evolution and recent divergent levels (Susanti et al., 2017; Bronstein et al., 2018; Sharma and Sampath, 2019). As a heterogeneous unique marker, it avails the crucial population genomic information about the extinct species and their closely related extant populations (Knaus et al., 2011). For species that are vulnerable and critically endangered to extinction it is an appropriate tool in designing desirable breeding and management programmes for purposes of conservation (Dovc et al., 2006; Susanti et al., 2017; Md-Zain et al., 2019). It guides in structuring up phylogenetic relationships between species (MdZain et al., 2019), building monophyletic molecular divergence (Bakar et al., 2014; Md-Zain et al., 2019) and genetic diversity between species (Susanti et al., 2017; Md-Zain et al., 2019). It provides an appropriate platform in phylogenetic studies where individuals can be identified and specificity of breeds characterized accordingly to accurately explain their genetic

relationships (Cai et al., 2007; Susanti et al., 2017). Studying the genetic diversity within and between the species requires a good understanding of their phylogeographic, intra-species and inter-species relationships which can be traced in the mitochondrial D-loop (Purwantini et al., 2013; Susanti et al., 2017; Yan et al., 2019).

CHAPTER THREE

3.0 METHODS

3.1 The Study Area

The study was carried out in the northern and southern rangelands in Kenya. The northern range land comprise the entire northern Kenya and covers several ecosystems (Ojwang' et al., 2017). The main ones being: the larger Ewaso-Nyiro ecosystem, Terrestrial Coastal ecosystems and North eastern ecosystem. The North eastern ecosystem covers Mandera, Garissa, Marsabit and Isiolo and the Terrestrial Coastal ecosystems cover the landscapes in Lamu, Tana River and Kilifi. The southern rangeland cover the larger Maasai Mara ecosystem, Nairobi National Park, Lake Nakuru, Naivasha, Elementaita and Eburu forest ecosystem, Amboseli-Western Kilimanjaro ecosystem and the greater Tsavo ecosystem. The Maasai Mara ecosystem consists of the Maasai Mara National Reserve which is managed by the county government and other community conservancies that are privately managed. These include the Mara Triangle, Ol-Kinyei, Naboisho, Olare Motorogi, Lemek, Ol Churro and Mara-North (Anon, 2014; Ojwang' et al., 2017). However, sampling was done in selected areas of the two rangelands. In the northern rangeland, sampling was done in Marsabit, Maralal, Garissa, Isiolo, Waso and Lewa while in the southern rangeland, sampling was done the Maasai Mara National Reserve and five privately managed conservancies including the Mara North, Naboisho, Olare Motorogi, Ol Kinyei and Olare-Naboisho (Figures 3.1).

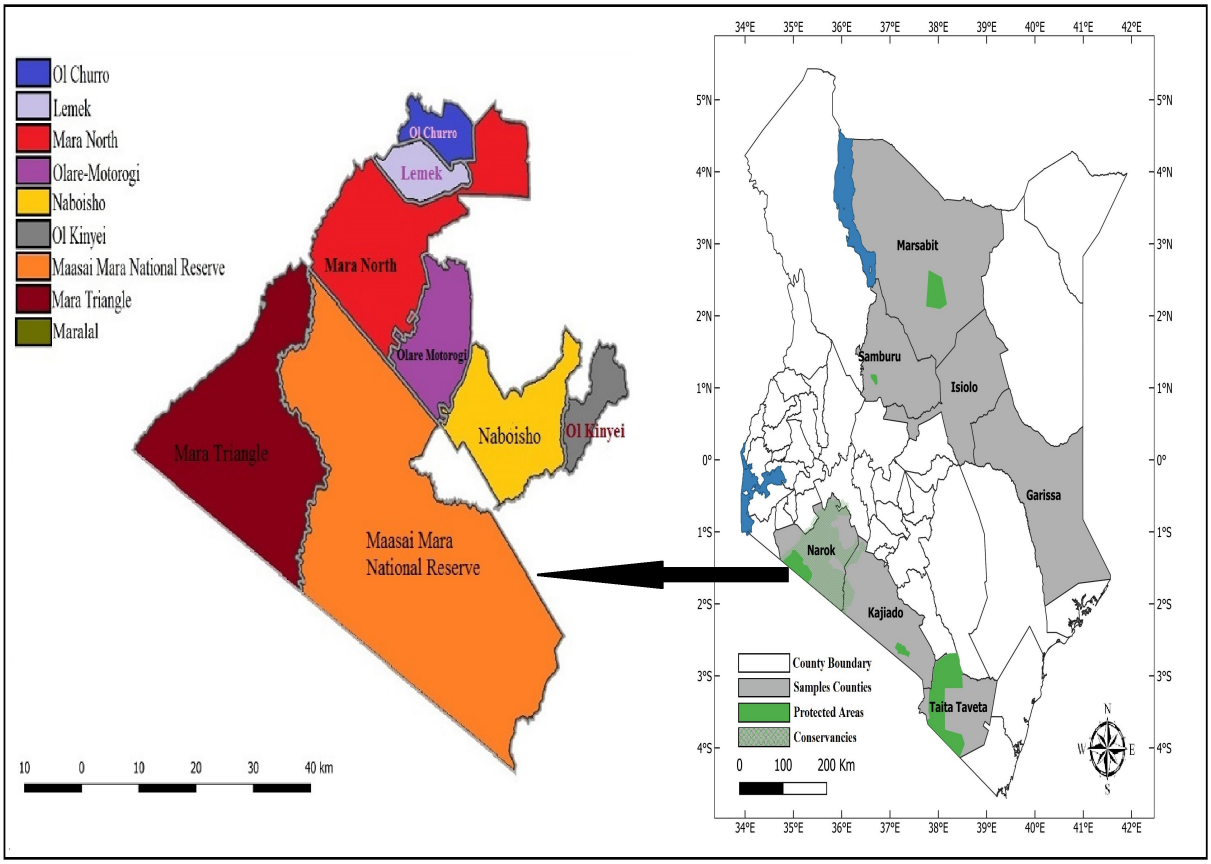


Figure 3.1: Kenyan Map showing areas of study. Samples were collected in areas with grey. Note that in Narok County, Maasai Mara NR and five private conservancies, Mara Triangle, Naboisho, Ol Kinyei, Mara North and Olare Motorogi where samples were collected.

3.1.1 Maasai Mara Ecosystem

The greater Maasai Mara ecosystem (GMME), a unique ecosystem located in South western Kenya lies between latitudes 1°, $15^{'}$ and 1°, $45^{'}$ to the South and longitudes 34°, $45^{'}$ and 35°, $25^{'}$ to the East. It covers the Maasai Mara National Reserve and several conservancies which are currently privately owned. The conservancies include: Mara Triangle, Mara North, Lemek, Ol Kinyei, Naboisho, Olare Motorogi and Ol Churro which forms the wildlife dispersal areas. The connection between the Maasai Mara Ecosystem and the Serengeti National Park of

Tanzania to the south forms the larger Serengeti-Mara Ecosystem which covers 25,000Km^2 between Northern Tanzania and Southern Kenya. The GMME is approximately 6,000 Km^2 with the MMNR covering approximately 1,510 Km^2 with the rest of the land about 4,490 Km^2 left as unprotected inhabited by the Maasai and other agro-pastoral communities (Ojwang' et al., 2017). The two protected areas cover more than 1.6 million hectares with MMNR accounting for 1,510 ha (approx. 373,000 acres) (United Nations Environment Program and World Conservation Monitoring Center).

The area is characterized by open rolling grasslands interspersed with shrubs and woodlands and has several permanent and seasonal rivers supplying the area with surface waters. The rainfall pattern is bimodal with the long rains from March to June and short rains from November to December each year. The rainfall patterns are greatly influenced by Intertropical Convergence Zones (ITCZ) with longer dry seasons between mid-June and mid-October and the shorter dry season between January and February. The Ecosystem is home to numerous ungulates including over 2 million migratory wildebeests (*Connochaetes taurinus*) and over 3,000 non migratory wildebeests, Zebras (*Equus burchelli*) and gazelles (*Gazella thomsoni*). This form part of the high density of prey biomass in the Ecosystem and supports a high density of predators such as the lion, leopard and several large mammals including elephants, buffalo and black rhinoceros. The migratory wildebeest and Zebras form a spectacular scene in the months of July and October or beyond, which makes this Ecosystem a major tourist attraction Centre in the country (Ojwang, 2012; Ogutu et al., 2016; Ojwang' et al., 2017).

3.1.2 Ewaso Ecosystem

The Ewaso ecosystem lie within the administration of Isiolo, Samburu, Laikipia, Wajir, Marsabit, Garissa and Meru counties in Northern Kenya (Ojwang' et al., 2017). However,

sampling was mainly done in conservancies located in Marsabit and Maralal counties. It extends from Mount Kenya slopes, South west of Aberdare Ranges, east of Turkana shoreline and to the north of Mount Marsabit. Most parts are arid and semi-arid (ASALs) with cultivation land, commercial pasture, ranches, wildlife protected areas and conservancies that define the ecosystem major land use. The area has altitude variations with Mount Kenya regions being 5,200 metres above sea level and 138 metres above sea level in Garissa county. The ecosystem has variable and unpredictable patterns of rainfall with most areas receiving rainfall in April and December every year. High areas around Mt. Kenya receive 1200 mm of rainfall per year while lower areas around Laikipia, most of Samburu and Isiolo get 300-600 mm of rainfall per year. Some areas of Garissa and Isiolo to the east receive an annual rainfall of less than 300 mm. In January to February and from June to September, majority of lower areas experience characteristic dry seasons. The rainfall patterns in Horr and Marsabit is bimodal having long rains in March to May and short rains in October to December. The Counties of Samburu and Laikipia highlands experience trimodal rainfall patterns and have long rains from April to June, short rains from October to December and another unpredictable rainy season between July and August (Ogutu et al., 2016; Ojwang' et al., 2017).

Approximately 90% of the land use in the ecosystem is livestock production in communal lands and wildlife conservation in private conservancies especially in Laikipia plateau. Most communal lands are unfenced and offer free movement of pastoralists, dispersal and migration of wildlife due to insecurity, drought and availability of pastures. However, privately owned land is fenced and thus hinders the movement of pastoralists and wildlife dispersal and

migration. Only 10% of Kenya's protected land is covered by National Parks and Reserves in Northern Kenya which provides habitat to Eastern African ungulates especially in Mara Serengeti ecosystem (Ogutu et al., 2016; Ojwang' et al., 2017).

3.2 Study Design

3.2.1 Ethical approval

Samples used in this study were previously donated to Institute of Primate Research (IPR) by KWS. Prior to this study, permission to work with these donated lion samples was granted by the IPR Institutional Ethical Review Committee (IERC) **Ref IERC/08/18** and by the Faculty of Veterinary Medicine Biosafety and Animal Care and Ethics Committee of the University of Nairobi **REF:FVM BAUEC/2018/159.**

INSTITUTE OF PRIMATE RESEARCH
NATIONAL MUSEUMS OF KENYA
WHO COLLABORATING CENTRE

NATIONAL MUSEUMS OF KENYA
WHERE HERITAGE LIVES ON

Our Ref IERC/08/18

Dear Dr. Joseph Kamau,

It is my pleasure to inform you that your proposal entitled "Genetic Characterization of AFRICAN LION (PANTHERA LEO MELANOCHAITA) POPULATIONS IN KENYA" in collaboration with David Thuo of Kenya Wildlife Trust and Dr. Daniel Chai of the Institute of Primate Research has been reviewed by the Institutional Review Committee (IRC) at a meeting of 28th June 2018. The proposal was reviewed on scientific merit and ethical considerations on the use of animal research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN, and Helsinki Convention on the humane treatment of animals for scientific for scientific purposes and GLP.

You are bound by the IPR Intellectual Property Policy.

Chairman, IRC

ADDRESS: P.O BOX 24481 (00502), KAREN, NAIROBI, KENYA - TEL: +254-02-882571/4 - FAX : +254-02-882546 – URL :www.primateresearch.org - EMAIL: info@primateresearch.org

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Mr Peter Irungu Maguta

C/o CEBIB'

Chiromo Campus

REF:FVM BAUEC/2018/159

27/07/2018

Dear Mr Irungu,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Mitochondrial DNA analysis of Eastern African lion (*Panthera leo melanochaita*) populations in Kenya

By Peter Irungu Maguta I56/81673/2015

We refer to the above MSc proposal that you submitted to the committee for review and approval. We have now reviewed your proposal and have noted that:

In this study, for your DNA analysis, you intend to use samples previously collected by KWS personnel from lions in different parks across the country. Kindly, liaise with the relevant department in KWS for permission to use the required specimens.

We hereby approve your study as detailed in your proposal.

Rodi O. Ojoo BVM M.Sc. Ph.D.

Chairman, Biosafety, Animal Use and Ethics Committee,

Faculty of Veterinary Medicine

3.2.2 Study subjects

A total of 120 lions were sampled in this study including 26 samples from the northern frontier, 90 samples from the southern frontier, the Maasai Mara ecosystem and 4 samples from unknown location (Figures 3.2-3.3). Out of total samples, 58 DNA samples did not amplify during the PCR reaction and out of the remaining 63 DNA samples that amplified, 7 were eliminated from the study due to quality of the sequenced data. A total of the remaining 56 DNA samples were used for genetic analysis in this study.

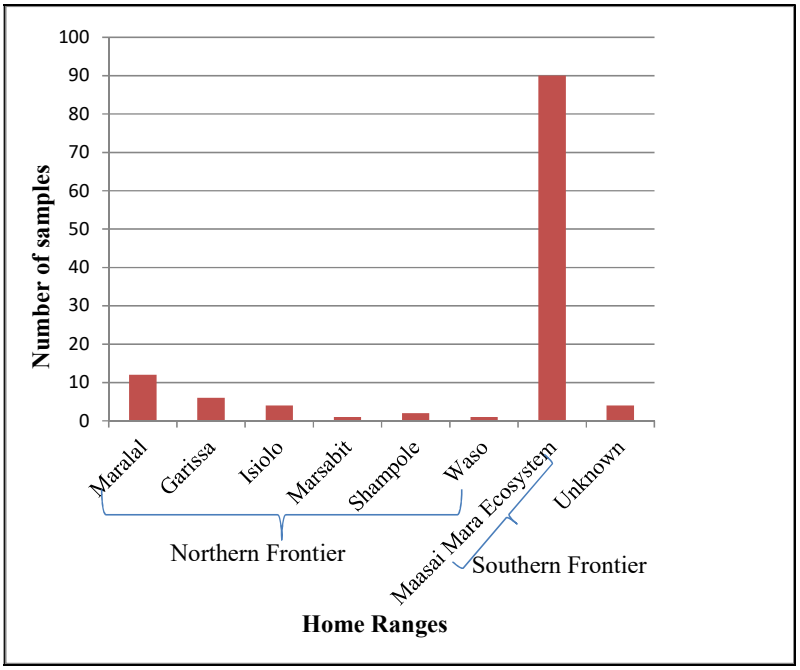

Figure 3.2: A Bar Chart showing the number of lions sampled in the individual Northern frontier home ranges and in the entire southern frontier in this study

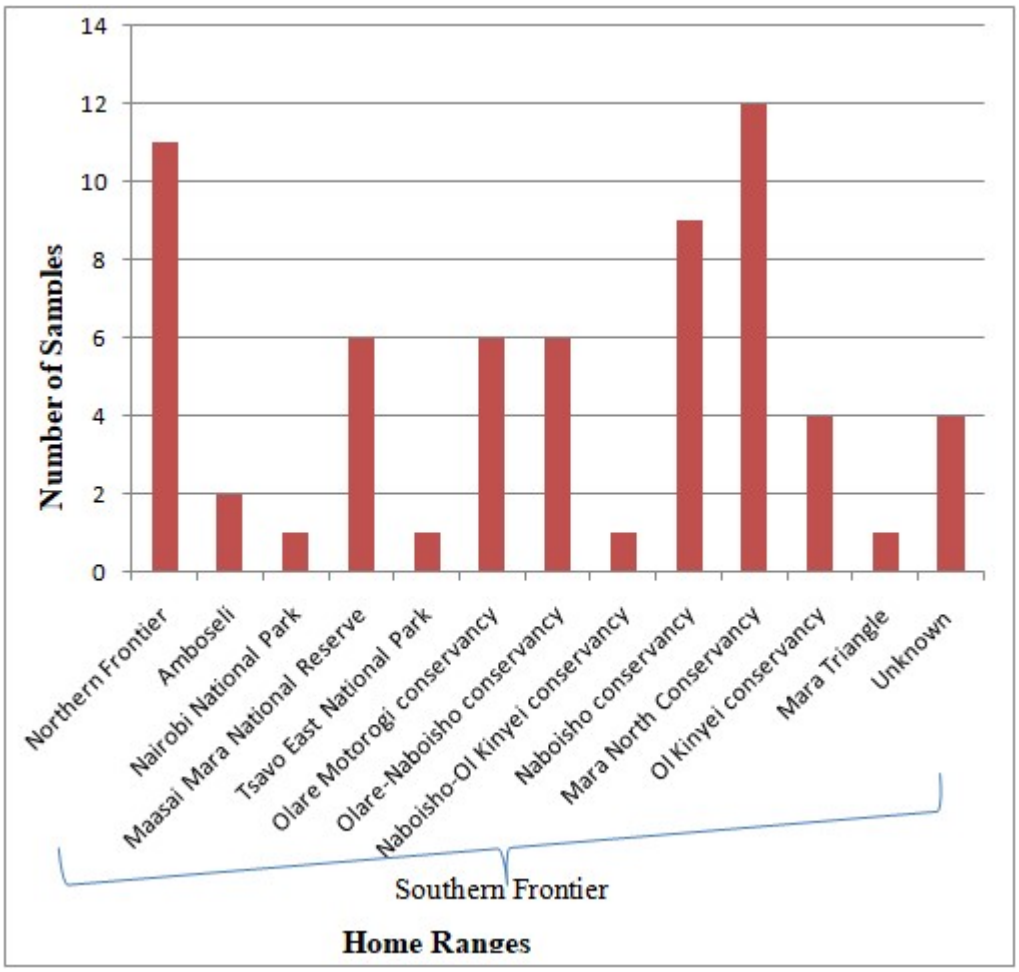

Figure 3.3: A Bar Chart showing the number of lions sampled in the entire Northern frontier and individual home ranges in the southern frontier in this study

3.2.3 Identification of individual lions

The lions used in this study were individually identified by use of whisker spots. Semi-automated whisker spot recognition software initially developed for bears was used. Whisker spot patterns of individual lions were taken by qualified wildlife guides and three reference points were manually selected in reference to the position of the mouth, eye and nose according to Anderson et al. (2010). The face of the lion was photographed and the quality of the photographs was enhanced by use of Adobe Photoshop version 7.0. These whisker spot

patterns were used to match individual lions against their photographs in the database. Three points at each whisker spot patterns were manually selected because the lions color changes with age and there was high contrast between the whisker spot patterns color and the lion's fur color. The three reference points included the outer end of the mouth, the inner corner of the eye and the corner of the nostrils. The spatial coordinates of the mouth, the eye and the nose as reference points were set up for comparing the whisker spot patterns of different lions as per Osterrieder, et al., 2015.

The software program standardized the spatial coordinates of the location of these points such that, the mouth was at (0.5, 0.5), the eye (0, 0) and the nose (1, 0). These coordinates were used to align the whisker spots of different individuals.

Figure 3.4: Whisker spot patterns for identification of different lion faces A, B and C, Source: (Osterrieder et al., 2015)

3.2.3.1 Age Estimation

Aging is an important global measuring system for management research and sustainable harvest of several wildlife species including lions. It helps in assessing wildlife history including population size, reproduction and movement (White et al. 2016; Miller et al. 2016). Due to the history for the African lions population decline, age estimation is a crucial tool to understand lions' population dynamics (Lindsey et al., 2013b; White et al., 2016). It aids to

clarify the species life tables as indicated by specific age of the animal (Miller et al., 2016; White et al., 2016). It is also an important tool in management for monitoring the population trends (White et al., 2016) and possible effects of the causes of death of the species (Frank et al., 2005; Whitman and Packer, 2007; White et al., 2016).

In this particular study, sighted lions were closely approached and examined for identification and estimation of their ages by qualified veterinarians and wildlife guides using a 4x4 wheel drive Range Rovers. Young cubs were aged using their ability to stand, walk, color of the eye and nose tip and their coat texture. Adult lions were aged in to four different age classes including one to two years, three to four years, five to six years and above seven years characterized by changes in mane development, facial scarring, teeth color and wear (White et al., 2016), jaw slackness and nose pigmentation (Figure 2.2) (Miller et al., 2016). This was summarized in the tables below.

Table 3.1: Age estimation by mobility, eye colour, nose tip colour, coat colour, tail tuft colour and mane development.

AGE	MOBILITY	EYE COLOUR	NOSE TIP COLOUR	COAT COLOUR	TAIL TUFT	BODY SIZE	FACIAL MARKINGS	MANE DEVELOPMENT
≥3 Weeks	Ability to stand & walk	-	-	-	-	-	-	-
5-6 Weeks	Ability to follow the mother	-	-	-	-	-	-	-
6-7 Weeks	Self dependent	-	-	-	-	-	-	-
> 8 Weeks	Ability to follow the pridal family	-	-	-	-	-	-	-
2-3 Months	-	Grayish Blue			-	-	-	-
3 Months	-	-	Light grey or Pink	Woolly	-	-	-	-
5 Months	-	-	Light grey or Pink	Adult-like in texture	Tail tuft appears	-	-	-
7 Months	-	-	Light grey or Pink	-	Tail tuft prominent and evident	-	-	-
1-2 Years	-	-	Light grey or Pink	-	-	2/3 Size Of Female Lions, Males larger than females, taller than mothers	-	Mane around the neck longer /darker
3-4 Years	-	-	freckled with tiny spots	-	-	-	-	Mane around the neck longer /darker
5-6 Years	-	-	Freckled join and liver spotted	-	-	-	Facial fur duller and thin with age, lion looks darker above the muzzle	Mane fills the neck, chest & shoulder blade
7-8 Years	-	-	Nose completely black	-	-	-	-	Mane loose condition, hair frazzle, some loose mane length
8-9 Years	-	-	-	-	-	-	Muzzle hair thinner, whisker sports less visible	-
> 9 Years	-	-	-	-	-	-	Whisker sports visible	-

Table 3.2: Lion aging by dentition (Tooth eruption, X-ray pulp cavity and chamber closure, tooth colour and crown wear)

Age	Tooth Eruption	X-Ray Pulp Cavity and Chamber Closure	Tooth colour	Crown Wear
15-17 Months	All permanent teeth appear except canines	At two years and four months to three years, canines' closure of apical foramina seen, happens earlier in female lions than in male lions, lower canine apical foramina closes before the upper canine apical foramina	White	Teeth sharp and in color
3-4 years	All permanent teeth appear	Wide cavity at the age of 3 years, 3 months, pulp cavity continues to close and fills up at the age of 4 years and 9 months	-	Small wear on incisors, canines and premolars
5-6 Years	All permanent teeth present	-	Canine yellow, longitudinal ridge	Obvious wear on canines, incisors, 3rd upper premolar and lower 4th premolar with little wear on 2nd upper premolar and 3rd lower premolar
7-9 Years	All permanent teeth present	-	Distal ridge completely worn out, all teeth have some degree of yellowness,	All teeth show obvious wear, canines and incisors prominently worn out, incisors show most wear or might be missing
≥ 9 Years	All permanent teeth present	-	Yellowing becomes evident in all the teeth	Obvious wear in all the teeth, canine teeth broken or missing, one or more incisors worn down to the stump or entirely missing

3.2.3.2 sex determination

To estimate the sex of the young lions, the presence or absence of mane, size of the canine teeth and the size of *temporalis* musculature were used. Male lions have mane, larger canine teeth and muscle sizes as a sign for successful hunting and biting force. Female lions have no mane, have small canine teeth and small muscle sizes.

3.2.4 Sample Collection

3.2.4.1 Lion immobilization and physical examination

The Pneudart biopsy darts customized for African lions were used for darting lions during immobilization. The female lions were darted at the deep muscle of the shoulder and the male lions at the thigh for immobilization. Males were darted at the thigh because of the presence of mane around the neck region which hinders the procedure to be carried out efficiently. Lions were immobilized by sedating them with a combination of Teletamine/Zolazepan (Telazol) and Xylazine in a ratio of 250mg/200mg for males and 167mg/133mg for females or a combination of medetomidine and ketamine. The Medetomidine (Dormitor) is a sedative drug that acts as alpha 2 agonist whereas ketamine is a short acting cyclohexylamine knock down drug. Whenever Teletamine/Zolazepan (Telazol) and Xylazine were administered, 10 mg/kg body weight Teletamine, 5 mg/kg body weight Zolazepan and 1.5 mg/kg Xylazine dosages were used. When Medetomidine and Ketamine were administered, a dosage of 50 µg/kg body weight Medetomidine and 5 mg/kg body weight Ketamine were used. Medetomidine is a sedative and analgesic alpha-2 adrenoceptor adrenergic agonist that potentiates the effects of other anaesthetic drugs such as Ketamine. The effects of medetomidine were reversed with antipamezole which is a specific, selective and potent alpha-2 adrenoceptor antagonist drug at a dosage of 0.25 mg/kg body weight. The animals recovered from anesthesia after one and a half hours. After immobilizing the animals, physical examination of individual lions was conducted to assess the well-being of the lion before samples were collected.

3.2.4.2 Blood sample withdrawal and processing

Fresh blood samples were collected from immobilized lions or opportunistically when lions were being treated. Blood samples from the jugular vein in females and from radial femoral

vein in males were then collected in sterile vacutainer tubes containing Ethylene Diamine Tetra-acetic Acid (EDTA) anticoagulant (Dubach et al., 2013) which were previously well labled with animal number, sex, age, location and date of collection. This is because the presence of mane in males hinders blood collection from the jugular vein. The blood samples were then packaged in a cool box with ice cubes for preservation before transit. The samples were then transported in a van to KWS and then to Institute of Primate Research for processing, DNA extraction and subsequent analysis.

On arrival at the IPR laboratories, whole blood samples which were collected in EDTA vacutainer tubes were centrifuged at 1,500 rpm for 15 minutes to increase the yield of nucleated cells. The buffy coat was then harvested in cryovials of Tris EDTA Buffer (100mM Tris, 100mM EDTA and 2% SDS at pH 8.0) and stored at -20°C until the DNA was extracted.

3.2.4.3 Skin Tissue Samples collection and processing

Skin tissue samples were collected by remote biopsy darting method or opportunistically during treatment, radio collaring, de-snaring or when a veterinarian or wildlife guides encountered a dead lion (Dubach et al., 2013; Mijele et al., 2016). The candidate lion was located by the local knowledge of wildlife guides using call up vocalization at kills. In call-up vocalization, an array of sounds like those of a dying wildebeest (*Connochaetes*) calf were used as per Ogutu and Dublin (1998) to attract lions to a station in order to dart them (Elliot and Gopalaswamy, 2017). At least one female in a pride were previously radio collared and this was used to locate members of the same pride since they move together. The lions were approached to approximately 40-50 meters using a vehicle; the males and female lions were then darted as previously described. On impact the biopsy darts ferrule tip cut and extracted approximately 1/8″ x 3/16" x 5/15" skin tissue sample before falling off the animal. When the

dart was recovered, the cutting ferrule was unscrewed from the dart to reveal tissue in the barbed pin of the dart body. All sample collection containers were well labled with as previously described. The tissue samples were then transferred to a sterile cryovials containing absolute ethanol (100%) or Tris-buffer in cool boxes, transported to KWS and then to the Institute of Primate Research molecular laboratory for processing, DNA extraction and subsequent genetic analysis. Skin tissue samples that were preserved in absolute ethanol (100%) or Tris-buffer for transit were, after delivery, stored at -20°C at the IPR molecular laboratories until the DNA was extracted.

3.2.5 Laboratory assays/methods

3.2.5.1 DNA Extraction.

DNA was extracted using the Pure Link Genomic DNA purification MiniKit (Invitrogen) Catalogue number K1820-02 manufactured in USA. The kit was suitable for extracting genomic DNA from several sources including mammalian tissues and blood samples. Extraction of DNA using the kit was achieved by selective binding of DNA to Silica-based membrane in the presence of chaotropic salts. The contents of the kit included 50 ml Genomic Elution Buffer (10 mM Tris-Hcl, pH 9.0 and 0.1 mM EDTA), 37.5 ml Genomic Wash Buffer 2, 50 ml Genomic Wash Buffer 1, 45 ml Genomic Digestion Buffer and 50 ml Genomic Lysis/Binding Buffer. Other kit components included 5.0 ml Proteinase K in storage buffer, 5.0 ml RNase A (20 mg/ml) in 10 mM EDTA and 50 mM Tris-Hcl, pH 8.0), 5 x 50 Spin Columns with 2.0 ml collection tubes and 5 x100 collection tubes (2.0 ml). Before the procedure began, all reagents were assembled and put at room temperature. The Genomic Wash Buffer 1 and 2 were diluted with Absolute Ethanol following manufacturer's guidelines.

3.2.5.2 DNA extraction from blood

Extraction of genomic DNA from blood started by preparation of the cell lysate where 200 microliters of whole frozen blood sample pre-collected in EDTA was added in a 1.5 ml sterile microcentrifuge tube. Then 20 microliters of Proteinase K was added to digest and lyse the blood cells for optimal enzymatic activity. This was followed by addition of 20 microliters of RNase A to degrade the RNA present in the sample and to minimize contamination of the purified DNA by RNA. The mixture was briefly vortex mixed and incubated for 2 minutes at room temperature. After incubation, 200 microliters of Genomic Lysis/Binding Buffer was added and was well mixed by vortexing to obtain a homogeneous solution. To enhance complete digestion of proteins, the homogeneous solution of the lysate was incubated in a water bath pre-set at 55°C for 10 minutes. Then 200 microliters of Absolute ethanol was added to the lysate and then vortex mixed for 5 seconds to homogenize the solution and allow for optimal binding of the DNA.

To bind the DNA, about 640 microliters of the lysate was added to the Spin columns and centrifuged for 1 minute at 10,000 x g. The flow-through was discarded together with the collection tube. Then the Spin column was placed in clean sterile collection tubes and the washing of DNA was initiated. The DNA was washed with 500 microliters of Wash Buffer 1 (with 75 ml Absolute Ethanol pre-added). This was added to the Spin columns and centrifuged for 1 minute at 10,000 x g in room temperature. The collection tube with the flow through was replaced with new collection tubes. With 87.5 ml of Absolute Ethanol added to PureLink Genomic Wash Buffer 2, 500 microliters of the Buffer was added, the solution was centrifuged for 3.0 minutes at room temperature at maximum speed (14,000 x g rpm). The collection tube was discarded and DNA elution started. To elute the DNA, the Spin Columns

were placed in clean sterile 1.5 microcentrifuge tubes. Then 50 microliters of Genomic Elution Buffer was added to the Spin Column. The mixture was incubated for 1 minute at room temperature and then centrifuged at maximum speed for 1 minute. The elution was repeated using new sterile collection tubes with the same elution volume to recover more DNA.

3.2.5.3 DNA extraction from skin tissue samples

To extract the DNA from skin tissue samples, skin tissue lysate was first prepared in a 1.5 ml sterile microcentrifuge tube by adding 25 mg of minced skin tissue. This was followed by addition of 180 microliters of Genomic Digestion Buffer and 20 microliters of Proteinase K to completely immerse the minced tissue. The mixture was well mixed and incubated for 4 hours at 55°C with intermittent vortexing until the tissue was completely digested. The lysate was centrifuged for 3 minutes at 14,000 x g rpm and the supernatant transferred to sterile 1.5 ml eppendorff tubes. To degrade and remove any RNA present that may contaminate the purified DNA, 20 microliters of RNase A was added into the mixture and incubated at room temperature for 2 minutes. Then, 200 microliters of Genomic Lysis/Binding Buffer was added and well vortex mixed. Finally 200 microliters of Absolute ethanol was added and then vortex mixed for 5.0 seconds to homogenize the solution and allow for optimal binding of the DNA.

To bind the DNA, about 640 microliters of the lysate was added to the Spin columns and centrifuged for 1 minute at 10,000 x g rpm. The flow-through was discarded together with the collection tubes. Then the Spin column was placed in a clean sterile collection tube and the washing of DNA was initiated. The DNA was washed with 500 microliters of Wash Buffer 1 (with 75 ml of Absolute Ethanol pre-added) and centrifuged for 1 minute at 10,000 x g rpm. The collection tubes with the flow through were replaced with new tubes. With 87.5 ml of Absolute Ethanol added, 500 microliters of diluted Genomic Wash Buffer 2 was added and

centrifuged for 3 minutes at room temperature at maximum speed (14,000 x g rpm). To elute the DNA, the Spin Columns were placed in sterile 1.5 eppendorff tubes. Then 50 microliters of Elution Buffer was added to the Spin Columns. The mixture was incubated for 1 minute at room temperature and then centrifuged at maximum speed for 1 minute. To recover more DNA, the elution step was repeated using new 1.5 ml sterile microcentrifuge tube with the same elution volume.

3.2.5.4 Measuring DNA quality and purity

The concentration and quality of DNA was measured in a Nano Drop 2000 Spectrophotometer (Thermoscientific,http://www.thermoscientifc.com/onebio). The absorbance of the DNA at wavelength 230, 260 and 280 nanometers (nm), its concentrations at 260/280 ratio and its purity at 260/230 ratio was analyzed in this study. The results of all the samples were listed in an excel spreadsheet. The normal DNA purity accepted as pure DNA at the ratio of 260/280 was 1.6 to 2.0. A value of less than 1.6 was interpreted as DNA contaminated with proteins, phenols and other components while a value of greater than 2.0 was interpreted as DNA contaminated with RNA. The ratio of 260/230 was interpreted to be the secondary measure of nucleic acid purity ranging from 1.8 to 2.2 while lower values were interpreted to be due to the presence of co-purified contaminants. The overall quality and purity of the used DNA in the present study was tested using seven samples and found to be good and satisfactory (Figure 3.5).

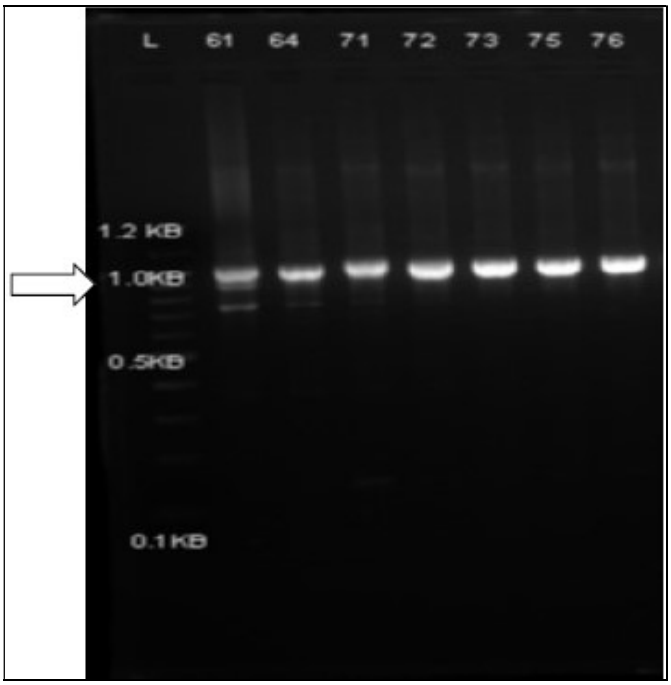

Figure 3.5: Showing the quality of DNA amplicons in Agarose gel with wells labled 61, 64, 71, 72, 73, 75 and 76 containing DNA amplicons of interest measuring 1.0 KB. Well labled L contain DNA ladder.

3.2.6 Polymerase Chain Reaction (PCR)

First, the PCR thermal cycler machine was well inspected to ensure good working condition and switched on prior to use. All other materials including PCR master Mix, DNA samples maintained at low temperatures in an ice pack were assembled ready for loading. The entire procedure was carried out in a well air conditioned and temperature controlled room for optimum functioning of the machine.

Both Forward (HCAT-F) and Reverse (HCAT-Phe-R) primers previously synthesized as per Dubach et al., 2005 at Inqaba Biotechnologies Limited, South Africa were used for the PCR reaction. The following sequences were used for the synthesis of both forward and reverse primers:

Forward Primer: HCAT-F: 5′ GCA TCT GGT TCT TAC TTC AGG 3′ and

Reverse Primer: HCAT-Phe-R: 5′ ATT TTC AGT GTC TTG CTTT 3′

The forward and reverse primers were optimized using the gradient PCR reaction in a gradient SimpliAmp thermal cycler from Applied Biosystems by Thermo Fisher Scientific in USA (SN 228007943). All the other conditions held at constant temperatures of 94 °C for 1 min (Initial denaturation), 94°C for 40 s (Second denaturation), 72 °C for 45 s (Initial extension) (35 cycles) and a final extension at 72 °C for 10 min, the optimum annealing temperature was found to be 52 °C which was also previously used by Dubach et al. (2005).

The target of both the forward and the reverse primer sequences in the PCR reaction of this study was 1000 bps mitochondrial D-loop region. The PCR reaction was carried out using 25µl PCR reaction master mix consisting of 18.15 µl pure nuclease free double distilled water, 2.5 µl of 10x PCR buffer, 0.75 µl of 50x $MgCl_2$, 0.5 µl of 10mM dNTPs, 1.0 µl of both forward and reverse primers, 0.1 µl of Taq polymerase and 1.0 µl (25ug) of DNA template added in a SimpliAmp Thermal Cycler from Applied Biosystems by Thermo Fisher Scientific (SN 228007943). Amplification of the 1000 bp mitochondrial D-loop region was done at the following conditions: 94 °C for 1 min (initial heating), 94 °C for 40 s, 52 °C for 45 s, 72 °C for 45 s (repeated for 35 cycles), 72 °C for 10 min (final extension) and the reaction was held at 4 °C.

For the best resolution of the 1000 bps long amplified PCR bands, 1.5% gel concentration was used for gel electrophoresis at a voltage of 100 for 2 hours.The PCR bands were then viewed in a gel documentation system and photographs of each gel were taken and saved in a

separate folder. The photographs for each gel were then edited using Windows 7.0 operating system snipping tool to its appropriate size and labled accordingly. After the gel was viewed and amplicons of 1000 bps identified, the bands were excised using a sterile scalpel blade and then purified and eluted using the GeneJET gel purification kit. The purified DNA was then used for Sanger sequencing.

3.2.7 DNA Sequencing

After DNA purification and elution, 25 µl out of the 30 µl of the purified DNA was packaged together with both forward and reverse primers and sent for ABI Sanger sequencing at Macrogen Company, Netherlands in North Western Europe (http://www.macrogen.co.uk). The Sanger sequencing method was used because it is the method of choice for sequencing nucleotides from small size projects and is a cost effective method. The 5 µl of the purified DNA was aliquoted and 2µl used for confirmatory PCR to ensure that the mtDNA D-loop was successfully purified and recovered or eluted for sequencing. The sequencing was done using both the Forward HCAT-F (5′ GCA TCT GGT TCT TAC TTC AGG 3′) and Reverse HCAT-Phe-R (5′ ATT TTC AGT GTC TTG CTTT 3′) primers.

3.2.8 Submission of DNA to GenBank for Accession Numbers

Following the sequencing of the 56 DNA samples, the consensus DNA sequences were submitted to GenBank (Submission ID: 2452480) at National Centre for Biotechnology Information to be assigned Accession Numbers. The Fasta format of the DNA consensus sequences were submitted by use of BankIt GenBank submission tool as plain text and results obtained after three working days. The following is a table of Accession Numbers for the 56 DNA consensus sequences as assigned by GenBank (Table 3.3).

Table 3.3: Showing the Accession Numbers (MW987592- MW987647) assigned to the 56 consensus DNA sequences used in this study using the BankIt submission tool in GenBank at the National Centre for Biotechnology Information

S/No	Sample ID	Accession Number	S/No	Sample ID	Accession Number	S/No	Sample ID	Accession Number
1	Lio120	MW987592	20	Lio94	MW987611	39	Lio49	MW987630
2	Lio118	MW987593	21	Lio93	MW987612	40	Lio48	MW987631
3	Lio117	MW987594	22	Lio92	MW987613	41	Lio45	MW987632
4	Lio116	MW987595	23	Lio91	MW987614	42	Lio43	MW987633
5	Lio115	MW987596	24	Lio87	MW987615	43	Lio41	MW987634
6	Lio114	MW987597	25	Lio86	MW987616	44	Lio39	MW987635
7	Lio112	MW987598	26	Lio76	MW987617	45	Lio38	MW987636
8	Lio111	MW987599	27	Lio75	MW987618	46	Lio32	MW987637
9	Lio110	MW987600	28	Lio73	MW987619	47	Lio30	MW987638
10	Lio109	MW987601	29	Lio72	MW987620	48	Lio29	MW987639
11	Lio107	MW987602	30	Lio71	MW987621	49	Lio16	MW987640
12	Lio106	MW987603	31	Lio64	MW987622	50	Lio12	MW987641
13	Lio105	MW987604	32	Lio61	MW987623	51	Lio10	MW987642
14	Lio104	MW987605	33	Lio59	MW987624	52	Lio9	MW987643
15	Lio103	MW987606	34	Lio57	MW987625	53	Lio8B	MW987644
16	Lio99	MW987607	35	Lio55	MW987626	54	Lio8	MW987645
17	Lio98B	MW987608	36	Lio54	MW987627	55	Lio6	MW987646
18	Lio98	MW987609	37	Lio53	MW987628	56	Lio1	MW987647
19	Lio95	MW987610	38	Lio51	MW987629			

3.3 Data Analysis

3.3.1 Mapping of Contig Reads to Reference Sequence

The sequenced results were downloaded from the Macrogen website as a.b.i trace files in a zipped folder. The a.b.i trace files containing sequenced data in form of chromatograms were extracted, organized and saved in a separate folder named as LIO sequenced data. The 14-day free trial Geneious Prime software version 2019.2.3 was downloaded from the Geneious website to be used for Contig mapping to generate consensus sequences, editing consensus sequences, trimming of multiple sequence alignments and DNA sequence analysis. The mitochondrial DNA Control Region, 1,668 bp, of the African lion (Kenyan Isolate) Gene Bank Accession Number KP001498 was downloaded from the NCBI in the Geneious Prime platform. This was used as the Reference sequence for mapping Contig reads of sequenced data. Both Forward and Reverse sequences of each of the sequenced 63 samples were mapped to 1,668 bp Reference Sequence of mitochondrial control region of *Panthera leo* Kenyan isolate (Figure 4.8). These were used to generate consensus DNA sequences of approximately 1000 bps long that were then edited in Geneious prime and aligned in MEGA X software using multiple sequence alignment program ClustalW.

3.3.2 Alignment of Consensus Sequences

A total of 56 consensus DNA sequences approximately 1000 bps long that were previously mapped in Geneious Prime version 2019.2.3 were exported in Fasta file format. The consensus DNA sequences were then uploaded and aligned in MEGA X software using ClustalW program (Figure 4.9). They were then exported, uploaded and trimmed in Geneious Prime version 2019.2.3 to obtain consensus sequences of 932 bps alignment of the same length and used for genetic analysis.

3.3.3 Genetic analysis

All the 56 consensus DNA sequences that were previously trimmed to 932 bps long were uploaded in bioinformatics softwares including the DNA Sequence Polymorphism (DnaSP) version 6.12.03, MEGA X and Geneious Prime Version 2019.2.3/20.0.05 for genetic analysis. The analysis performed included the determination of mitochondrial DNA polymorphisms, estimation of genetic variations and evaluation of maternal lineages.

The mitochondrial DNA polymorphisms for the entire population and sub populations were evaluated using the (DnaSP) software version 6.12.03. These included the analysis of polymorphic sites, analysis of Insertion-Deletion (In-Del) polymorphism, analysis of haplotype distribution and diversity, analysis of pair wise and per site DNA polymorphisms, analysis of number of mutations, analysis of nucleotide diversity and average number of nucleotide differences. Analysis of polymorphic sites involved analysis of the total number of polymorphic (variable) sites, the number and distribution of singleton variable sites and parsimony informative sites (Figure 4.10, Table 4.1). Analysis of pair wise and per site DNA polymorphism involved analysis of nucleotide differences and nucleotide diversity (Figure 4.11, Table 4.2). Analysis of InDel polymorphism involved the analysis of the number of InDel sites and InDel diversity per site pi(i) (Figure 4.12, Table 4.3). Analysis of haplotype distribution involved analysis of the total number of haplotypes (H) and haplotype diversity (Hd) (Figure 4.13).

Estimation of mean genetic diversities and nucleotide diversities was conducted in MEGA X and DnaSP Version 6.12.03 Softwares respectively. Genetic variations in mean genetic diversities (within subpopulation and in entire population) and mean genetic distances (within

group and between group) was conducted in MEGA X. Genetic variations in the total number of polymorphic sites, their distributions in to singleton variable and parsimony informative sites, InDel polymorphism, pair wise and per site DNA polymorphism, mutations, nucleotide diversities, average number of nucleotide differences, nucleotide divergence, gene flow and genetic differentiation was conducted in DnaSP Version 6.12.03. To estimate the mean genetic diversities and mean genetic distances, nucleotide divergence between populations, gene flow and genetic differentiation, the entire population was organized in to thirteen groups and each group had two sub populations based on the region of sampling, conservancy and pride. Based on the region of sampling, the northern and the southern frontiers were analyzed. Based on the conservancy, the Maasai Mara National Reserve, Mara North, Naboisho, Olare Motorogi, Olare/Naboisho and Ol Kinyei were considered while based on the pride, Engoyanai, Enesikiria, Cheli and Moniko prides were analyzed.

Analysis of maternal lineages of the fragmented lion population in Kenya was conducted in MEGA X Software using the phylogenetic inference of the aligned consensus DNA sequences with accession numbers (MW987592-MW987647). Phylogenetic trees were generated using MEGA X software and Maximum Likelihood method. This study used three species of *Panthera* family as out-groups for rooting the phylogenetic trees including the jaguar (*Panthera Onca*) Accession Number: KP483864, tiger (*Panthera Tigris*) Accession Number: DQ151550 and leopard (*Panthera Pardus*) Accession Number: KP001507. Three nucleotide substitution models including the Kimura-2 parameter model, Tamura-Nei nucleotide substitution model (Tamura and Nei, 1993) and Hasegawa-Kishino-Yano (HKY) nucleotide substitution model (Hasegawa, Kishino and Yano, 1985) were used. The three nucleotide substitution models assume different rates of nucleotide substitutions, different equilibrium

base frequency for each base and different rates of transition to tranversions evolution (Hasegawa, Kishino and Yano, 1985). The original trees, original scaled trees and bootstrap consensus phylogenetic trees were drawn under complete deletion option for gaps and bootstrapping at 1000 replicates for quality of the phylogenies (Figures 4.17-4.25).

CHAPTER FOUR

4.0 RESULTS

4.1 Samples analyzed

Out of the total 120 sampled lions from the northern and southern frontiers of Kenya, 63 samples were sequenced and results for 56 samples that gave good a.b.i chromatograms were analyzed. Below are bar-graphs to indicate the number of samples from each location that were actually analyzed.

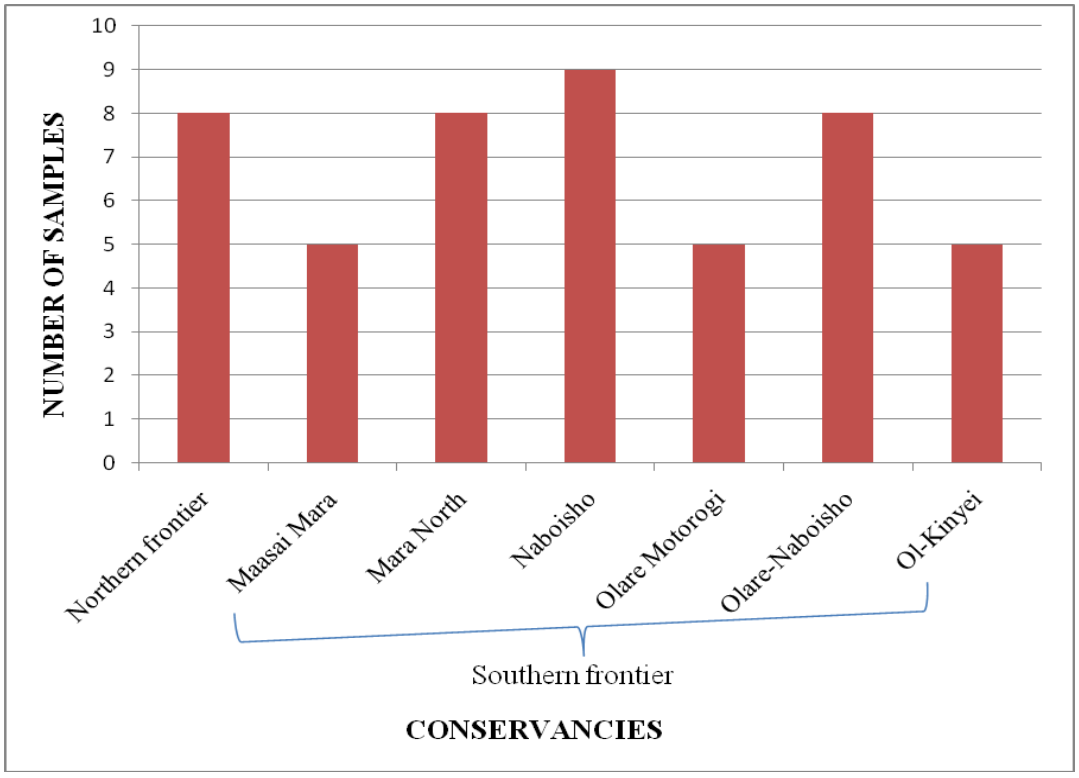

Figure 4.1: A Bar Chart showing the number of lion samples analyzed in the entire Northern frontier and individual home ranges in the southern frontier in this study

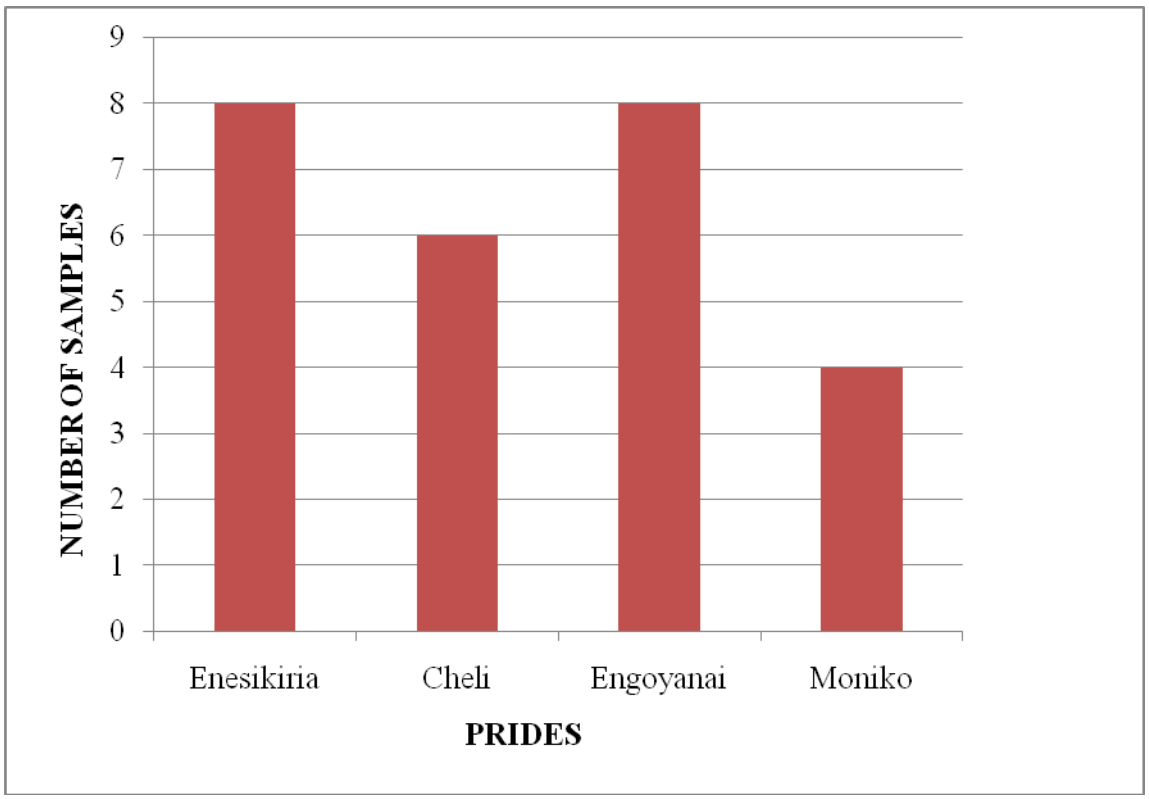

Figure 4.2: A Bar Chart showing the number of lion samples analyzed in the four lion Prides in the southern frontier in this study

4.2 Results for PCR

The results for the amplified bands were displayed as shown in the figures below (Figures 4.1-4.5):

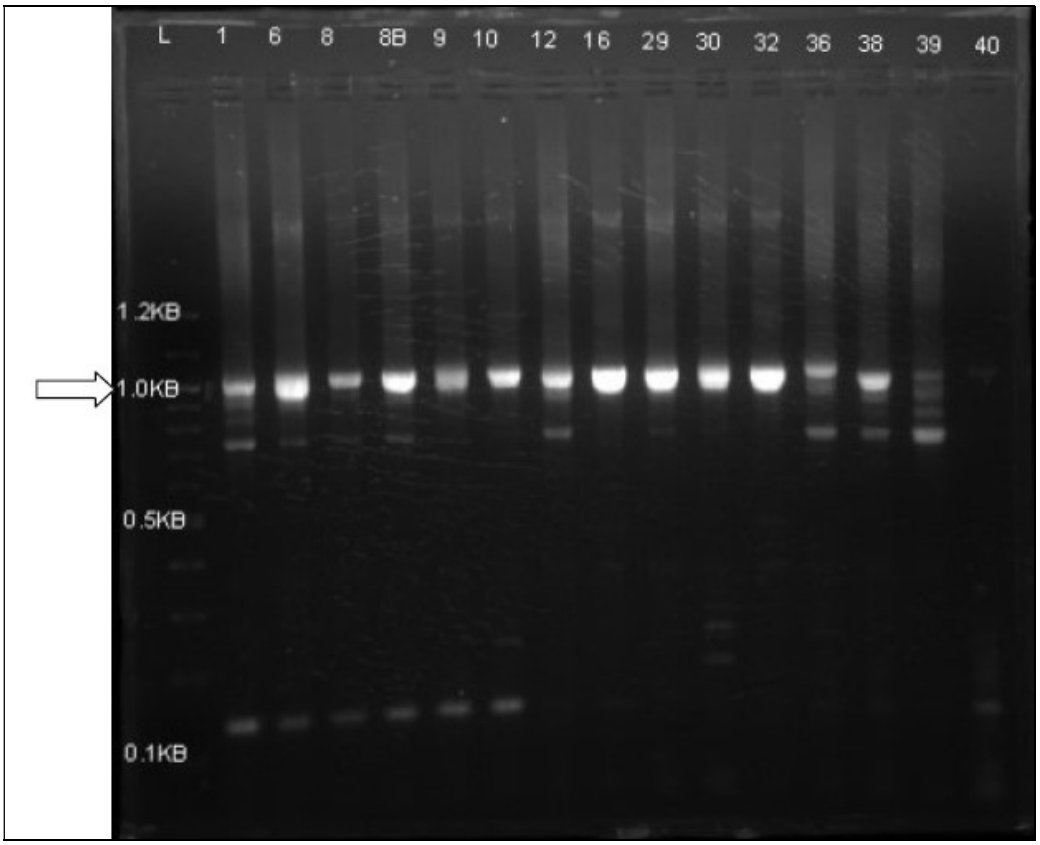

Figure 4.3: Showing Agarose gel 1 with multiple DNA amplicons of 1.0KB, 0.75 KB and 0.15 bp. Well labled L contain DNA ladder, Wells labled 1, 6, 8, 8B, 9, 10, 12, 16, 29, 30, 32, 36, 38, 39 and 40 contains DNA samples

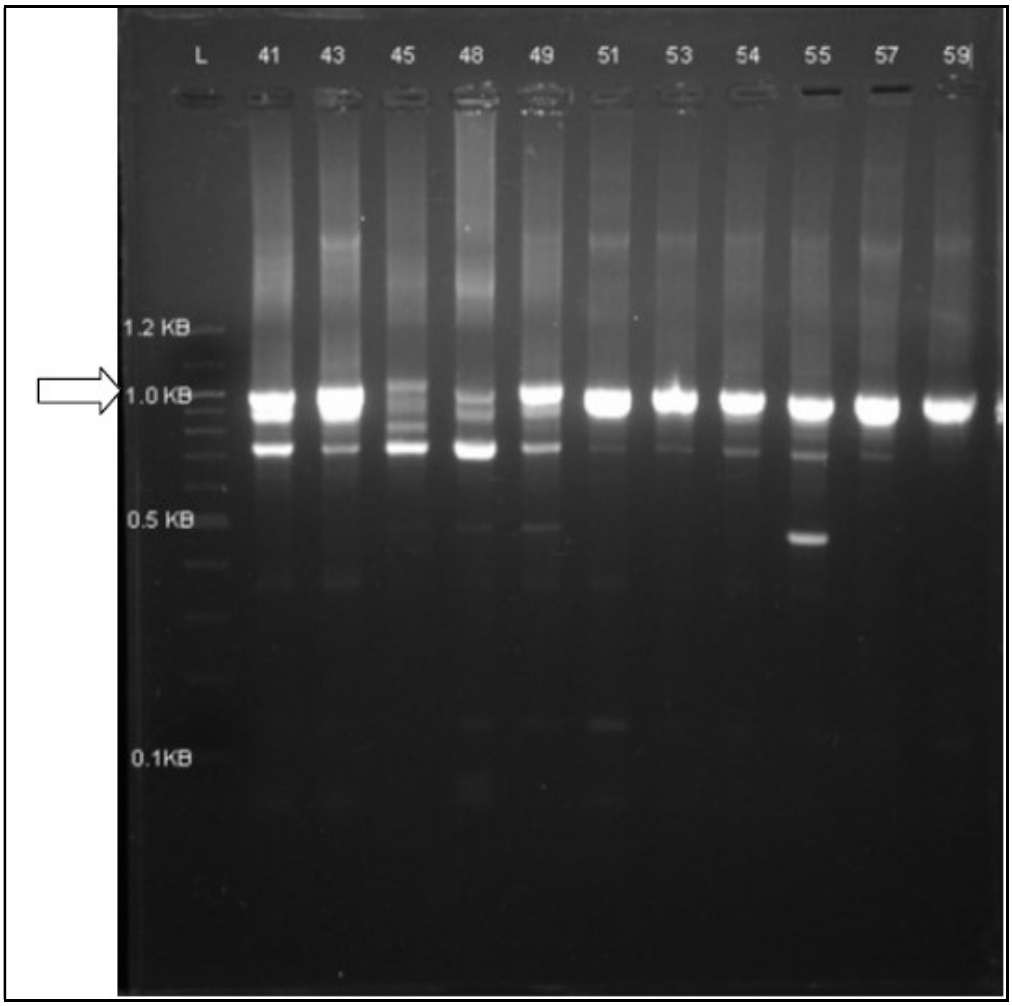

Figure 4.4: Showing Agarose gel 2 with multiple DNA amplicons of 1.0 KB,0.75 KB and 0.5 KB. Well labled L contain DNA ladder, Wells labled 41, 43, 45, 48, 49, 51, 53, 54, 55, 57 and 59 contains DNA samples

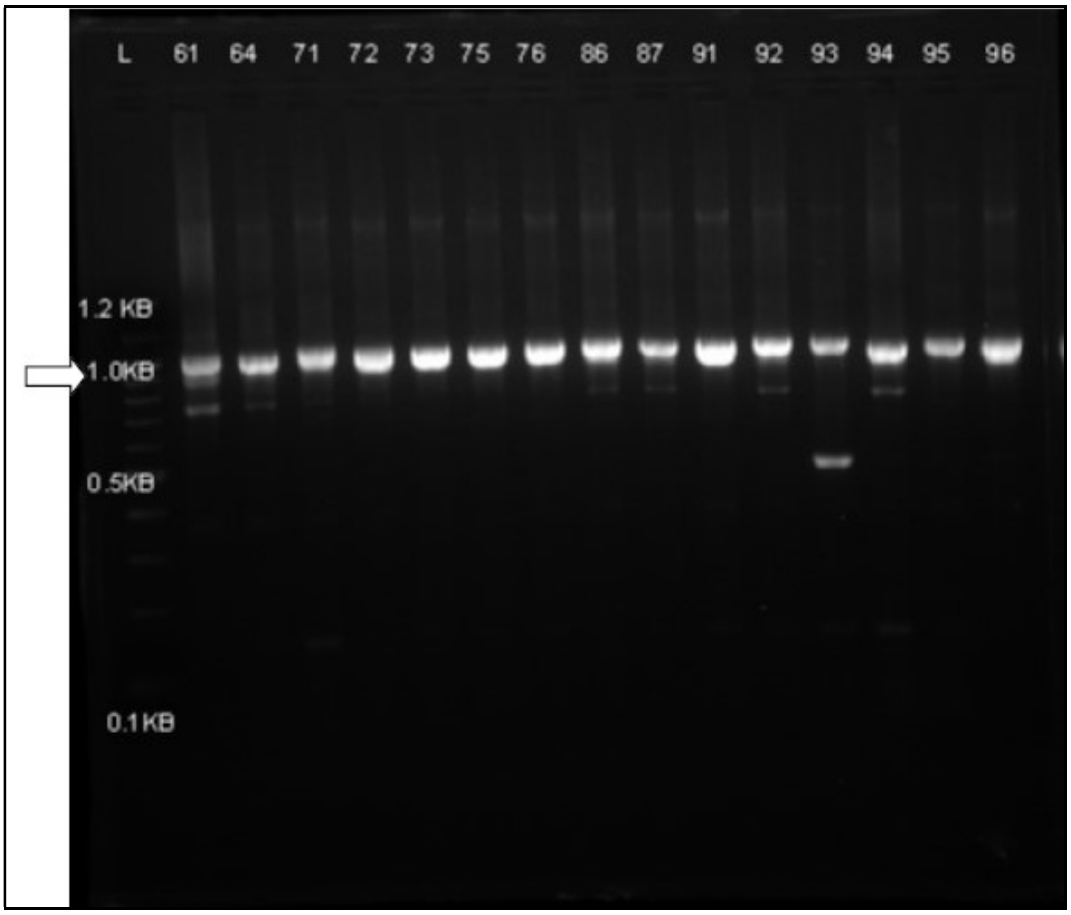

Figure 4.5: Showing Agarose gel 3 with multiple DNA amplicons of 1.0 KB, 0.75 KB and 0.5 KB. Well labled L contain DNA ladder, Wells labled 61, 64, 71, 72, 73, 75, 76, 86, 87, 91, 92, 93, 94, 95 and 96 contains DNA samples

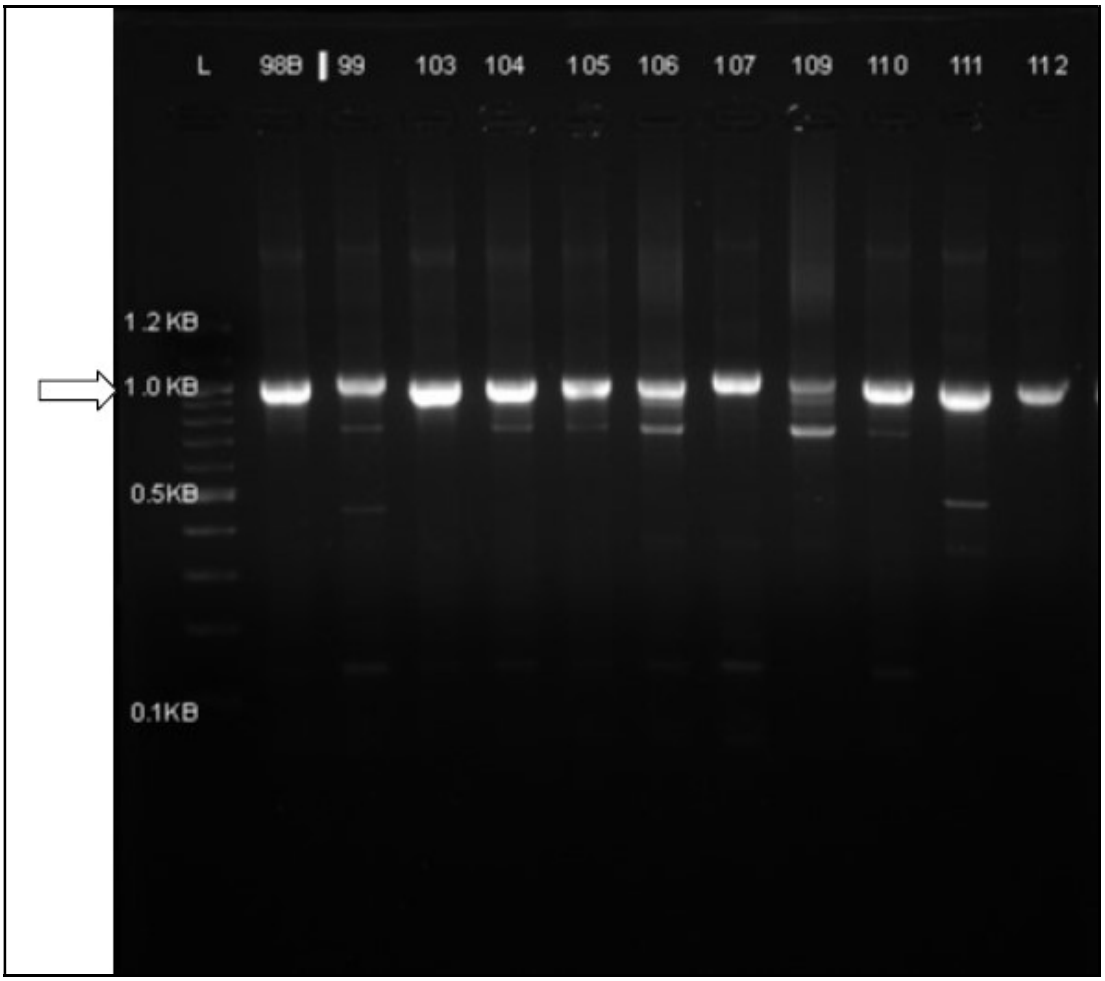

Figure 4.6: Showing Agarose gel 4 with multiple DNA amplicons of 1.0 KB, 0.75 KB, 0.5 KB and 0.15 KB. Well labled L contain DNA ladder, Wells labled 98B, 99, 103, 104, 105, 106, 107, 109, 110, 111 and 112 contains DNA samples

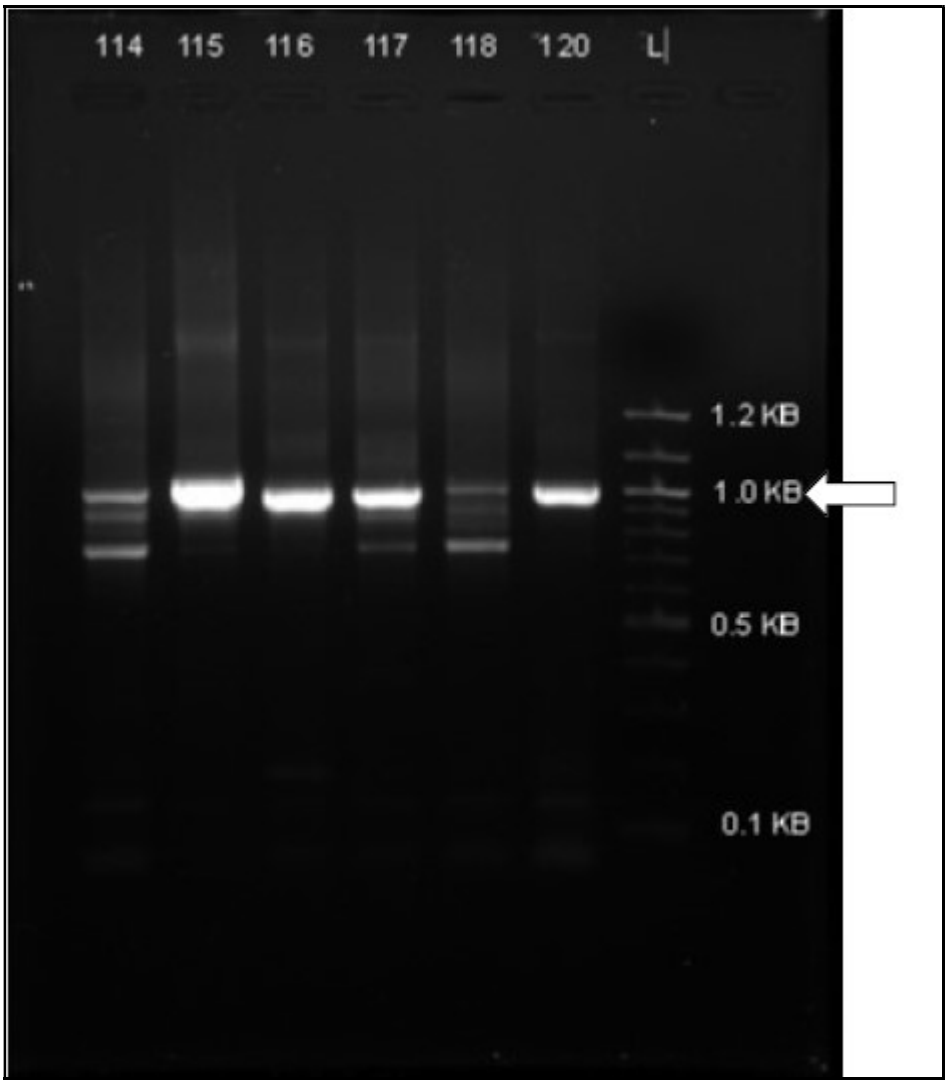

Figure 4.7: Showing Agarose gel 5 with multiple DNA amplicons of 1.0 KB and 0.75 KB. Well labled L contain DNA ladder, Wells labled 114,115,116,117, 118 and 120 contains DNA samples

4.3 Results for Contig reads mapping

A section of results of consensus sequences generated from both forward and reverse Contig Reads after mapping to Reference Sequence is shown below (Figure 4.8).

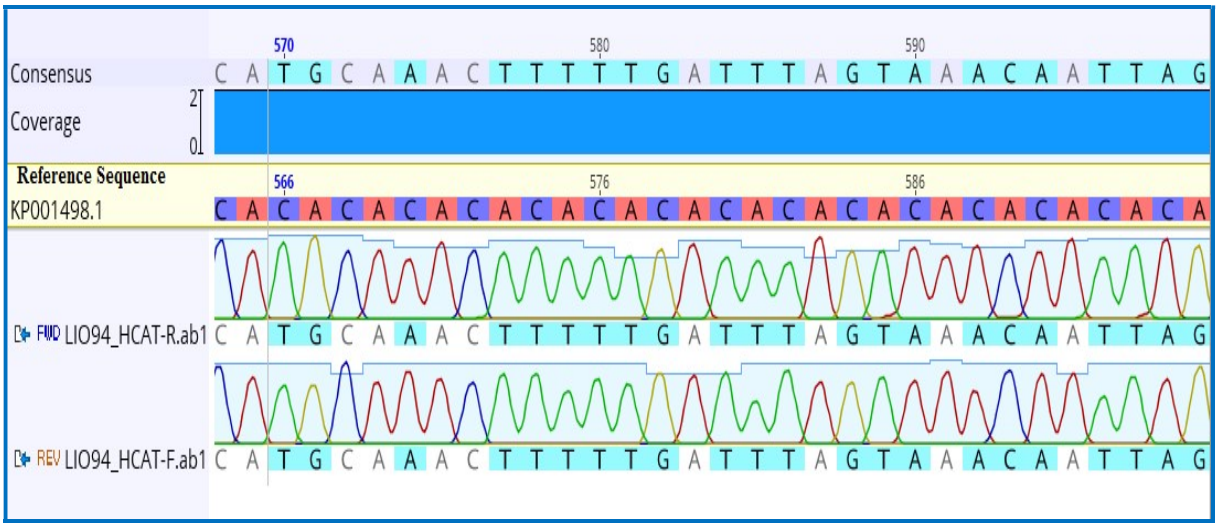

Figure 4.8: Showing the Consensus DNA sequence generated from the two Contig Reads of both forward and Reverse DNA sequences mapped to Kenyan lion mtDNA D-loop (Accession Number KP001498.1) as Reference Sequence of mitochondrial DNA control region of Kenyan Isolate. Mapping done in (Geneious Prime Version 2019.2.3)

4.4 Results for multiple sequence alignment of consensus sequences

Below is a section of results obtained after multiple sequence alignment of 56 DNA consensus sequences approximately 1000 bps long that were previously generated in Geneious Prime version 2019.2.3 aligned in MEGA X software using ClustalW multiple sequence alignment program (Figure 4.9).

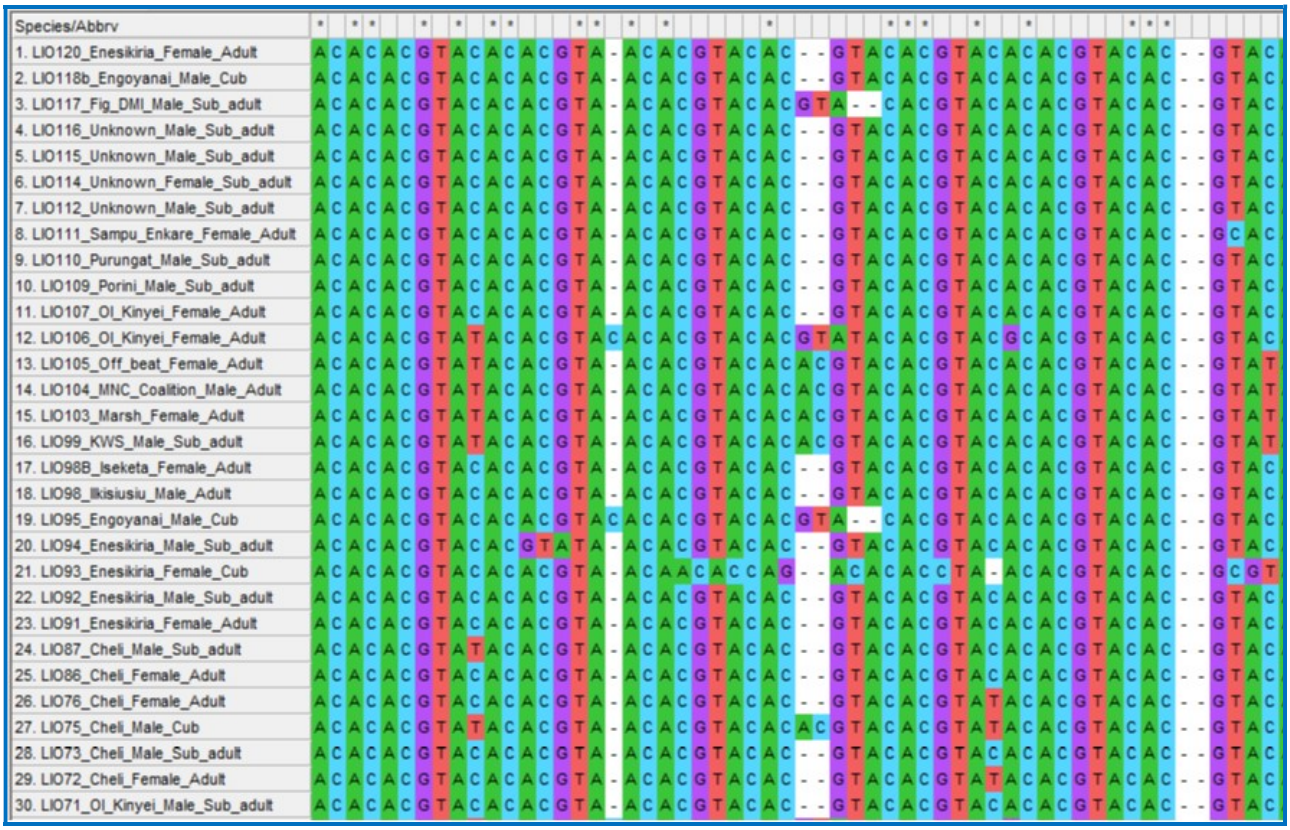

Figure 4.9: Showing part of Alignment file for the 56 lion DNA consensus sequences, Green: nucleotide Adenine (A), Blue: nucleotide Cytosine (C), Purple: nucleotide Guanine (G), Red: nucleotide Thymine (T) (Alignment done in (MEGA X))

4.5 Genetic Variations

In this study, we analyzed the variations in mean genetic diversity (within sub-populations and entire population) and mean genetic distances (within and between groups), InDel sites, pairwise and per site polymorphism, polymorphic sites, nucleotide diversity and average nucleotide differences of the northern frontier and the southern frontier lion populations and selected subpopulations.

Low level of mean genetic diversity (within sub-populations and in entire population diversity) was recorded in the entire lion population of approximately 0.052. Similarly, low level of between group mean genetic distances was recorded of approximately 0.05. The northern frontier population showed the highest within group mean genetic distance of 0.08 than the southern frontier lions which showed low within group mean genetic distance of 0.01. Slightly lower levels of mean genetic diversities and genetic distances were exhibited by the six lion conservancies in the southern frontier relative to the northern frontier lion population. The mean genetic diversity (within sub population and in entire population) between the northern frontier lions and lions in each of all the six individual conservancies ranged between 0.04 and 0.05.

The within group mean genetic distances of the northern frontier lion population ranged between 0.06 and 0.08 relative to the 0.01 within group mean genetic distances recorded in all the six individual southern frontier lion conservancies. The between group mean genetic distances between the northern frontier lion population and the six individual southern frontier ranged between 0.042-0.05. The highest between groups mean genetic distances recorded was between the northern frontier population and the Olare/Naboisho conservancy and the lowest was between the northern frontier population and Olare Motorogi conservancy.

Analysis of mean genetic diversity in the four selected prides showed that the mean genetic diversity within sub population ranged between 0.01-0.015 with the Enesikiria and Moniko group having the highest of 0.015. Analysis of their mean genetic distances indicated that the lions in Enesikiria pride are distantly related (high variation) with the highest within group mean genetic distance of between 0.02-0.025 and lions in the Moniko pride were very closely related (low variation) with the lowest within group mean genetic distance of 0.00. The lion population between Enesikiria and Engoyanai are distantly related (high variation) with the highest between group mean genetic distance of 0.0178 while lion between the Engoyanai and Moniko pride were very closely related (low variation) with the lowest between group mean genetic distances of 0.00715. (Table 4.4).

A close relationship was also observed between group mean genetic distances and nucleotide divergence of the two sub populations in each group. High between group mean genetic distances corresponded with the high levels of nucleotide divergence in each group. The between group mean genetic distances between the northern frontier and the southern frontier, between the northern frontier and the individual conservancies in the southern frontier ranged between 0.042 and 0.050 and the levels of nucleotide divergence ranged between 0.039 and 0.049. The within group mean genetic distances within the pride ranged between 0.007 and 0.018 and the nucleotide divergence ranged between 0.007 and 0.018 (Table 4.4).

Table 4.1: Showing the mean genetic diversity, mean genetic distances and nucleotide divergence. The mean genetic diversity (within sub-population and in entire population) and mean genetic distances (Within group and between groups) was analyzed in MEGA X software.

Group	Population	Within Group Mean Genetic Distance	Between Group Mean Genetic Distance	Mean Diversity within sub populations	Mean Diversity in Entire population	Mean Interpopulation Diversity	Nucleotide Divergence
Group 1	Northern Frontier Conservancy	0.080	0.0504	0.052	0.052	0.000	0.02156
	Southern Frontier Conservancy	0.012					
Group 2	Northern Frontier Conservancy	0.060	0.0416	0.040	0.040	0.000	0.04860
	Maasai Mara NR Conservancy	0.010					
Group 3	Northern Frontier Conservancy	0.080	0.0493	0.050	0.050	0.000	0.03952
	Mara North Conservancy	0.010					
Group 4	Northern Frontier Conservancy	0.080	0.0476	0.050	0.050	0.000	0.04847
	Naboisho Conservancy	0.010					
Group 5	Northern Frontier Conservancy	0.080	0.0502	0.050	0.050	0.000	0.04695
	Olare/Naboisho Conservancy	0.010					
Group 6	Northern Frontier Conservancy	0.060	0.0415	0.040	0.040	0.000	0.04900
	Olare Motorogi Conservancy	0.010					
Group 7	Northern Frontier Conservancy	0.060	0.0431	0.040	0.040	0.000	0.03910
	Ol Kinyei Conservancy	0.010					
Group 8	Engoyanai Pride	0.010	0.0178	0.010	0.020	0.000	0.04096
	Enesikiria Pride	0.020					
Group 9	Engoyanai Pride	0.010	0.0110	0.01	0.010	0.000	0.01779
	Cheli Pride	0.010					
Group 10	Engoyanai Pride	0.010	0.00715	0.010	0.010	0.000	0.01144
	Moniko Pride	0.000					
Group 11	Enesikiria Pride	0.025	0.0137	0.015	0.010	0.000	0.00715
	Moniko Pride	0.000					
Group 12	Enesikiria Pride	0.010	0.00836	0.01	0.010	0.000	0.01372
	Cheli Pride	0.010					

This study also estimated genetic variations in polymorphic sites (S), mutations (Eta), polymorphic mutations, Haplotype diversity (Hd), nucleotide diversity (Pi) and average number of nucleotide differences (k) for the entire lion population and sub-populations (Table 4.5). The entire lion population exhibited a mitochondrial D loop region with 282 polymorphic sites, 302 mutations with 249 being polymorphic, haplotype diversity of 0.9643, nucleotide diversity of 0.02103 and an average number of nucleotide differences of 17.3688. The results showed that the northern frontier lions had the highest number of polymorphic sites (S=234), mutations (Eta=241), polymorphic mutations (229), haplotype diversity of 1.00, nucleotide diversity (Pi=0.082) and the average numbers of nucleotide differences (k=68.29) compared to the southern frontier lions. The lion sub-populations in the Maasai Mara ecosystem (Maasai Mara NR and five private conservancies) in the southern frontier showed low number of polymorphic sites with a range of (S=11-16), mutations (Eta=11-16), polymorphic mutations (6-14), nucleotide diversity (Pi=0.0055-0.00896) and average nucleotide differences of between (k=6.39-11.54). Among these sub-populations, the Mara north conservancy showed the highest nucleotide diversity (Pi=0.00896) and average nucleotide differences (k=11.54). The three among the four lion prides studied in the southern frontier lion population exhibited low number of polymorphic sites ranging between (S=5-12), mutations (Eta=5-12), polymorphic mutations (3-11), nucleotide diversity (Pi=0.00311-0.0077) and average nucleotide differences between (k=4.67-8.83). The Enesikiria pride exhibited exceptional high levels of polymorphic sites (S=66), mutations (Eta=66), polymorphic mutations (66), nucleotide diversity (Pi=0.022) and average nucleotide differences between (k=27.8). The lions in the Enesikiria pride and Cheli pride among the four selected prides showed the most polymorphic mitochondrial D loop and the lions in Moniko pride showed the least polymorphic mitochondrial D loop.

Table 4.2: Polymorphic sites, mutations, haplotype and nucleotide diversity and average nucleotide differences per sub-population (Analysis done in DnaSP Version 6.12.03)

Populations	Polymorphic sites (S)	Mutations (Eta)	Polymorphic Mutations	Haplotype diversity (Hd)	Nucleotide Diversity (Pi)	Average Nucleotide difference (k)
Northern and Southern	282	302	249	0.9643	0.02103	17.3688
Northern Frontier	234	241	229	1.000	0.082	68.29
Southern Frontier	27	31	20	0.950	0.0113	9.34
Maasai Mara NR	11	11	9	1.000	0.0055	10.6
Naboisho Conservancy	37	37	9	1.000	0.00617	6.39
Mara North Conservancy	15	15	6	0.929	0.00896	11.54
Olare Motorogi Conservancy	15	15	11	1.000	0.00671	9.2
Olare Naboisho Conservancy	11	11	9	0.857	0.0066	8.18
Ol Kinyei Conservancy	16	16	14	1.000	0.00838	9.3
Enesikiria Pride	67	66	66	1.000	0.022	27.8
Cheli Pride	12	12	11	0.933	0.0077	8.83
Engoyanai Pride	10	10	9	0.917	0.0065	8.4
Moniko Pride	5	5	3	0.833	0.00311	4.67

Genetic variations in genetic differentiation and gene flow among the lion population in Kenya was also estimated in DnaSP Version 6.12.03 software using the Wright's F statistic (F_{st}) which represent the genetic differentiation, genetic differentiation coefficient (G_{st}), $Gammaa_{st}$ and gene flow or gene migration (N_m). The F_{st} values of 0.05 and below were interpreted to be low differentiation, values between 0.05 to 0.25 being moderate differentiation and greater values above 0.25 were interpreted to be high differentiation (Liu et al., 2016). High F_{st} values correspond to low N_m values and vice versa indicating that highly differentiated population has experienced limited chances of gene flow or gene migration while low differentiated population has free gene migration or movement (Liu et al., 2016). There were slightly low levels of gene flow (N_m=3.996) between the northern and the southern frontier population which also indicated differentiation coefficient (Fst=0.0660) in this particular study. Gene flow between the northern and the five selected private individual conservancies in the southern frontier was also observed to be slightly low ranging from (N_m=1.27-3.36) with differentiation coefficient range of (Fst=0.0693-0.1650). The northern frontier population and the Naboisho conservancy showed the highest level of gene flow (N_m=3.36) and the differentiation coefficient of (Fst=0.0693) while the northern frontier and the Ol Kinyei conservancy showed the lowest level of gene flow (N_m=1.27) and high differentiation (Fst=0.1650) among the private conservancies in this study. The highest level of gene flow (N_m=17.4) was observed among the lion populations in the Enesikiria and Moniko prides which also exhibited the lowest levels of genetic differentiation (Fst=0.0617). The lowest level of gene flow (N_m=0.48) or migration was observed among the lion population in the Engoyanai and Cheli prides which also experienced the highest level of genetic differentiation (Fst=0.34). Although the Kenyan lion population may not be completely differentiated, there is relatively low levels of gene flow and growing levels of

differentiation observed in a number of populations in this study (Table 4.6) and should be a worrying trend.

Table 4.3: Showing the Genetic differentiation and gene flow estimates between populations. Genetic differentiation coefficient (G_{st}), Gamma statistic ($Gamma_{st}$), Wright's F statistic (F_{st}) and Gene flow or Gene migration (N_m).

GROUP	POPULATION	Genetic Differentiation and Gene Flow Estimates			
		G_{st}	$Gamma_{st}$	F_{st}	N_m
Group 1	Northern Frontier Conservancy	0.0128	0.1007	0.0660	3.996
	Southern Frontier Conservancy				
Group 2	Northern Frontier Conservancy	0.0000	0.1977	0.1626	1.29
	Maasai Mara NR Conservancy				
Group 3	Northern Frontier Conservancy	0.0182	0.1038	0.0721	3.22
	Mara North Conservancy				
Group 4	Northern Frontier Conservancy	0.0000	0.1023	0.0693	3.36
	Naboisho Conservancy				
Group 5	Northern Frontier Conservancy	0.0370	0.1175	0.09753	2.31
	Olare/Naboisho Conservancy				
Group 6	Northern Frontier Conservancy	0.0000	0.0189	0.1470	1.45
	Olare Motorogi Conservancy				
Group 7	Northern Frontier Conservancy	0.0000	0.1990	0.1650	1.27
	Ol Kinyei Conservancy				
Group 8	Engoyanai Pride	0.0370	0.1609	0.1740	1.19
	Enesikiria Pride				
Group 9	Engoyanai Pride	0.0059	0.2913	0.3414	0.48
	Cheli Pride				
Group 10	Engoyanai Pride	0.0006	0.2741	0.2400	0.81
	Moniko Pride				
Group 11	Enesikiria Pride	0.0435	0.1750	0.0617	17.4
	Moniko Pride				
Group 12	Enesikiria Pride	0.0170	0.1373	0.0898	2.53
	Cheli Pride				
Group 13	Cheli Pride	-0.0233	0.2670	0.2286	0.84
	Moniko Pride				

4.6 Mitochondrial DNA Polymorphisms.

The 932 bp region of the mitochondrial D loop of the entire lion population analyzed in this study had a GC content of 42.8%, pairwise identity 97.2% and 58.9% identical sites analyzed in Geneious Prime Version 2020.0.5 software. Analyzed data of polymorphic sites of the mitochondrial D loop in this study indicated that, out of the 932 sites in the region, 106 sites were either alignment gaps or missing data. The remaining 826 sites were monomorphic and polymorphic sites. Out of the 826 sites, there were 544 monomorphic (invariable) sites, 282 polymorphic (variable) sites and 302 mutations or nucleotide substitutions (Figure 4.8). Different levels of nucleotide variants within the variable sites informed the genetic polymorphisms based on their distributions as singleton variable sites (S) and parsimony informative sites (PIs) within this studied mtDNA D loop region. Singleton variable sites were interpreted as variable sites with only one different nucleotide occurring multiple times and parsimony informative sites as having more than one nucleotide difference occurring multiple times. It also contains two nucleotides but only two of them occur with a minimum of frequency of two and is crucial to show phylogenetic relationship.

Among the 282 variable sites in this region there were 211 (74.82%) singleton variable sites and 71 (25.18%) parsimony informative sites. Within these singleton variable sites, 195 (92.4%) sites had two nucleotide variants (divariant), 15 (7.1%) singleton variable sites with three nucleotide variants (tripplevariant) and 1 (0.5%) singleton variable site with four nucleotide variants (quadrivariant). Among the 71 parsimony informative sites, there were 68 (95.8%) parsimony informative sites with two nucleotide variants (divariant), 3 (4.2%) parsimony informative sites with three nucleotide variants (tripplevariant). The region had none of the parsimony informative sites which were four nucleotide variants. Among the 106

sites that had either fixed or missing gaps, 9 sites had fixed gaps while 97 sites were Insertion Deletion (InDel) sites (Figure 4.10).

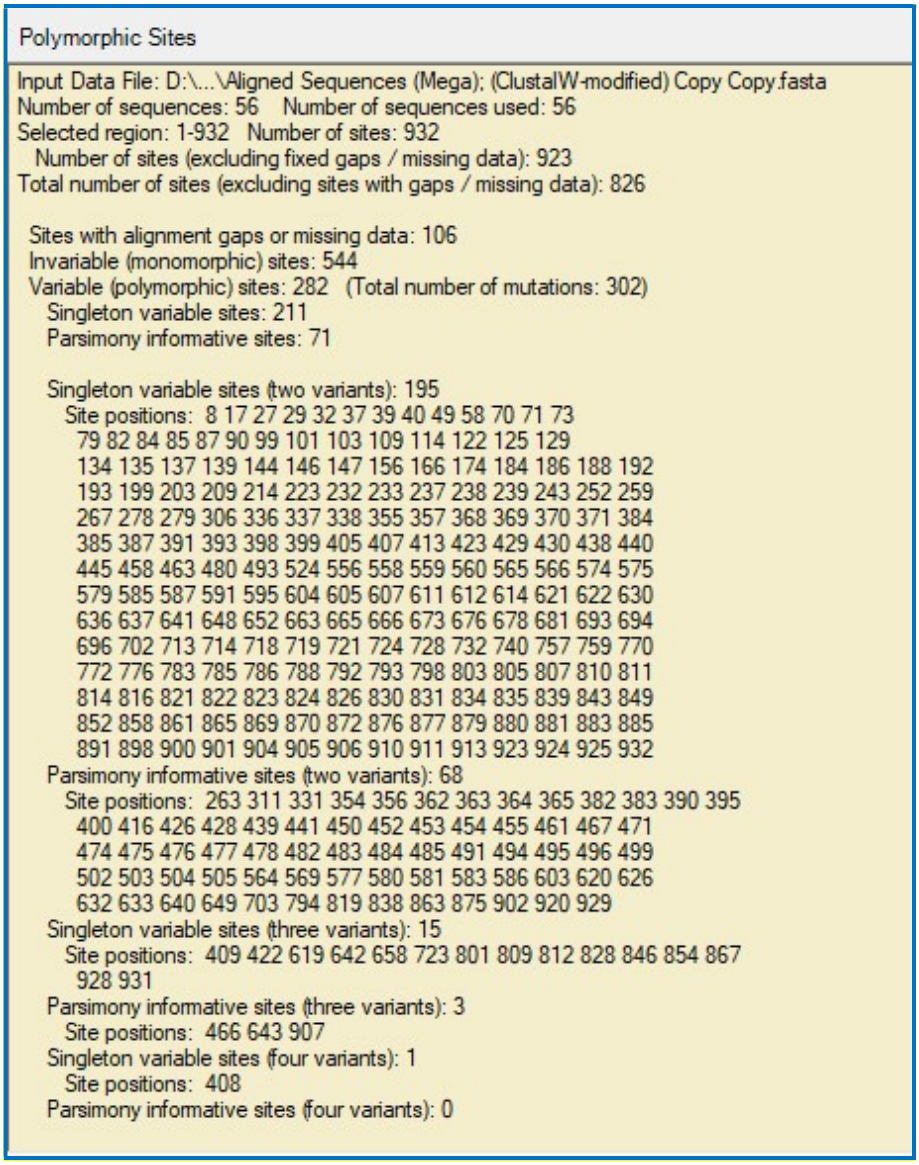

Figure 4.10: Showing the analysis and distribution of the polymorphic sites in the 932 bps region of the 56 mitochondrial D-loop of the Kenyan lions DNA Sequences. Analysis done in (DnaSP Version 6.12.03)

Among the two populations, the northern frontier population showed higher levels as compared to the Southern frontier population. The northern frontier lion population showed 234 variable sites and 241 mutations or nucleotide substitutions. Among the variable sites, there were 198 singleton variable sites and 36 parsimony informative sites. The singleton variable sites comprised 191 singleton variable sites with two nucleotide variants and 7 singleton variable sites with three nucleotide variants. The parsimony informative sites comprised 36 parsimony informative sites with two nucleotide variants only. The Southern frontier lions had 27 variable sites comprising of 24 singleton variable sites and 3 parsimony informative sites. Both the singleton variable sites and parsimony informative sites were divariant (Table 4.1).

Within the southern frontier population, we studied six conservancies in the Maasai Mara ecosystem including the Maasai Mara NR and other five selected private surrounding conservancies; the Mara North, Naboisho, Olare Motorogi, Olare Naboisho and Ol Kinyei. Among these conservancies, Ol Kinyei, Olare Motorogi, Naboisho and the Mara North had the most polymorphic mtDNA based on the number of polymorphic sites. However based on parsimony informative variable sites, the Mara North conservancy had the most parsimonious mtDNA D loop with the Olare Motorogi having the least parsimony informative mtDNA D loop (Table 4.1).

Among the four selected lion prides studied, the lion population in the Enesikiria pride showed 66 variable sites and 66 mutations. Among these variable sites, there were 61 singleton variable sites and 5 parsimony informative sites that had only two nucleotide variants. The lions in Cheli pride showed 12 variable sites and 12 mutations. They had 1 singleton variable site and 11 parsimony informative sites with only two nucleotide variants. The lions in

Engoyanai pride had 10 variable sites and 10 mutations. The population had 3 singleton variable sites and 7 parsimony informative sites which were only two nucleotide variants. The lions in Moniko pride had 5 variable sites and 5 mutations. The population had 4 singleton variable sites and 1 parsimony informative site and only two nucleotide variants. Polymorphism among the lions in the three prides was characterized by singleton variable sites and parsimony informative sites of two nucleotide variants only. Based on polymorphic sites among the four prides analyzed in this study, the mitochondrial D loop of Enesikiria pride was the most polymorphic followed by Cheli, Engoyanai, and Moniko pride which had 66, 12, 10 and 5 polymorphic (variable) sites respectively. However, among the four prides, the mitochondrial D loop of Cheli pride is the most parsimony informative followed by Engoyanai, Enesikiria and Moniko with 11, 7, 5 and 1 parsimony informative polymorphic sites (Table 4.1).

Table 4.4: Polymorphic sites, Singleton variable sites and Parsimony informative sites per sub-population (Analysis done in DnaSP Version 6.12.03)

Populations	Polymorphic Sites (S)	Singleton Variable Sites	S. Variable site variants	Parsimony Informative Sites	P. Informative Sites Variants
Northern and Southern	282	211	2 N Variants- 195 3 N Variants-15 4 N Variants-1	71	2 N Variants- 68 3 N Variants-3
Northern Frontier	234	198	2 N Variants- 191 3 N Variants-7	36	2 N Variants-36
Southern Frontier	27	24	2 N Variants- 24	3	2 N Variants- 3
Maasai Mara NR	11	8	2 N Variants-8	3	2 N Variants-3
Naboisho Conservancy	15	13	2 N Variants- 13	2	2 N Variants- 2
Mara North Conservancy	15	6	2 N Variants- 6	9	2 N Variants- 9
Olare Motorogi Conservancy	15	15	2 N Variants- 15	0	2 N Variants- 0
Olare Naboisho Conservancy	11	5	2 N Variants-5	6	2 N Variants- 6
Ol Kinyei Conservancy	16	11	2 N Variants- 11	5	2 N Variants- 5
Enesikiria Pride	67	61	2 N Variants-61	5	2 N Variants- 5
Cheli Pride	12	1	2 N Variants-1	11	2 N Variants- 11
Engoyanai Pride	10	3	2 N Variants- 3	7	2 N Variants- 7
Moniko Pride	5	4	2 N Variants- 4	1	2 N Variants- 1

Analysis of pairwise DNA polymorphism showed an average nucleotide difference of 18.5 and a nucleotide diversity of 0.02170. Analysis of DNA polymorphism per site showed an average number of nucleotide differences of 24.2 and a nucleotide diversity of 0.02746 (Figure 4.11).

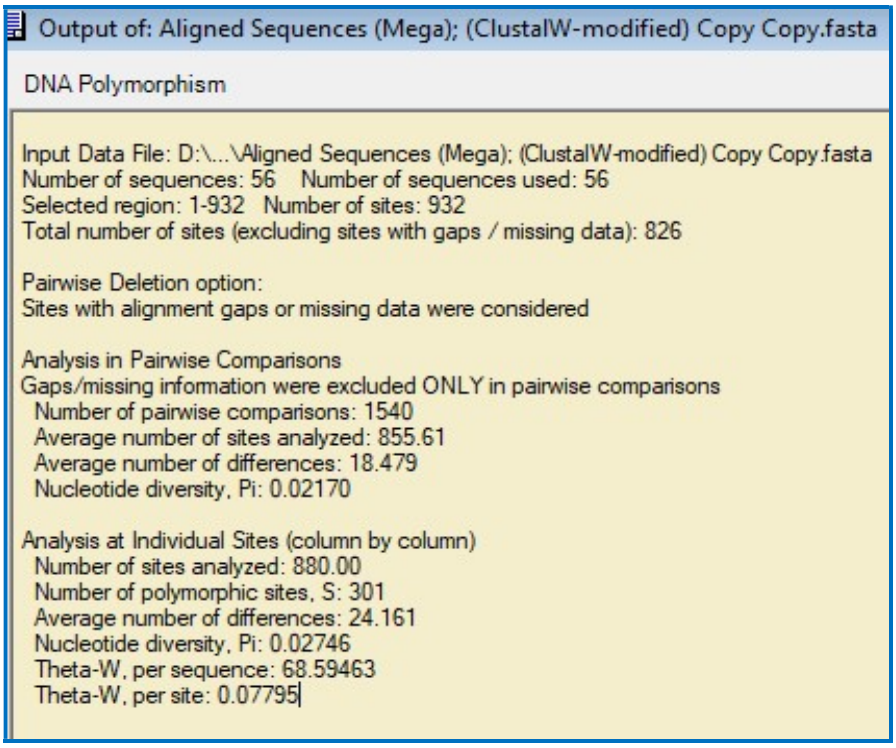

Figure 4.11: Showing analysis of DNA polymorphism in pairwise comparison and individual sites in the 932 bp region of mt. D-loop of Kenyan lion. Analysis done in (DnaSP Version 6.12.03)

The northern frontier lions showed the highest levels with an average nucleotide difference of 70.1 and a nucleotide diversity of 0.0828 in pairwise comparison analysis while analysis of polymorphism per site showed an average nucleotide difference of 73.114 and a nucleotide diversity of 0.0841 per site. The Southern frontier lions showed very low levels of 7.286 average nucleotide differences and 0.0085 nucleotide diversity in both pairwise analysis and

analysis per site (Table 4.2).

Among the six conservancies in the Maasai Mara ecosystem in the southern frontier, the Naboisho conservancy showed the highest polymorphism with an average nucleotide difference of 26.3 in both pairwise comparison analysis and per site analysis with a nucleotide diversity of 0.03077 in both cases. The second highest was Ol Kinyei with an average nucleotide difference of 7.7 and nucleotide diversity of 0.00899 in pairwise comparison analysis while per site analysis showed an average nucleotide difference of 7.9 and a nucleotide diversity of 0.00922. Mara North conservancy being the third highest had an average nucleotide difference of 7.5 and a nucleotide diversity of 0.00865 in both pairwise and per site analysis. The Olare Naboisho conservancy had the lowest levels with an average nucleotide difference of 5.0 and a nucleotide diversity of 0.0058 in both pairwise comparison analysis and per site analysis (Table 4.2).

Analysis of the four lion prides indicated that Enesikiria pride had the highest number of average nucleotide difference of 23.067 in both pairwise and per site analysis. It had a pairwise nucleotide diversity of 0.02705 and 0.02692 per site. The lions in this pride were followed by lions in Cheli pride with 6.6 average nucleotide differences and a nucleotide diversity of 0.0077 in both pairwise and per site analysis. The Engoyanai pride had 4.867 average nucleotide differences and a nucleotide diversity of 0.00568 in both pairwise and per site analysis. However, the Moniko pride had the lowest average nucleotide differences in both pairwise and per site of 2.667 and a nucleotide diversity of 0.00311 for both cases (Table 4.2).

Table 4.5: Showing analysis of DNA polymorphism in pairwise comparison and individual sites in the 932 bp region of mt. D-loop of Kenyan lion. Analysis done in (DnaSP Version 6.12.03)

S/NO	Population	Pairwise Comparison Polymorphism		Per Site Polymorphism	
		Average Nucleotide difference (k)	Nucleotide diversity (Pi)	Average Nucleotide difference (k)	Nucleotide diversity (Pi)
1	**Northern + Southern**	18.5	0.02170	24.2	0.02746
2	**Northern Frontier**	70.1	0.0828	73.114	0.0841
3	**Southern Frontier**	7.286	0.0085	7.286	0.0085
4	**Maasai Mara NR**	5.4	0.00630	6.333	0.00737
5	**Naboisho Conservancy**	26.3	0.03077	26.3	0.03077
6	**Mara North Conservancy**	7.5	0.00865	7.5	0.00865
7	**Olare Motorogi Conservancy**	6.0	0.00700	6.0	0.00700
8	**Olare Naboisho Conservancy**	5.0	0.0058	5.0	0.0058
9	**Ol Kinyei Conservancy**	7.7	0.00899	7.9	0.00922
10	**Enesikiria Pride**	23.067	0.02705	23.067	0.02692
11	**Cheli Pride**	6.6	0.00770	6.6	0.00770
12	**Engoyanai Pride**	4.867	0.00568	4.867	0.00568
13	**Moniko Pride**	2.667	0.00311	2.667	0.00311

The analysis of Insertion-Deletion (In-Del) sites among the whole population revealed 97 In-Del sites and an In-Del diversity per site of 0.00282 (Figure 4.12).

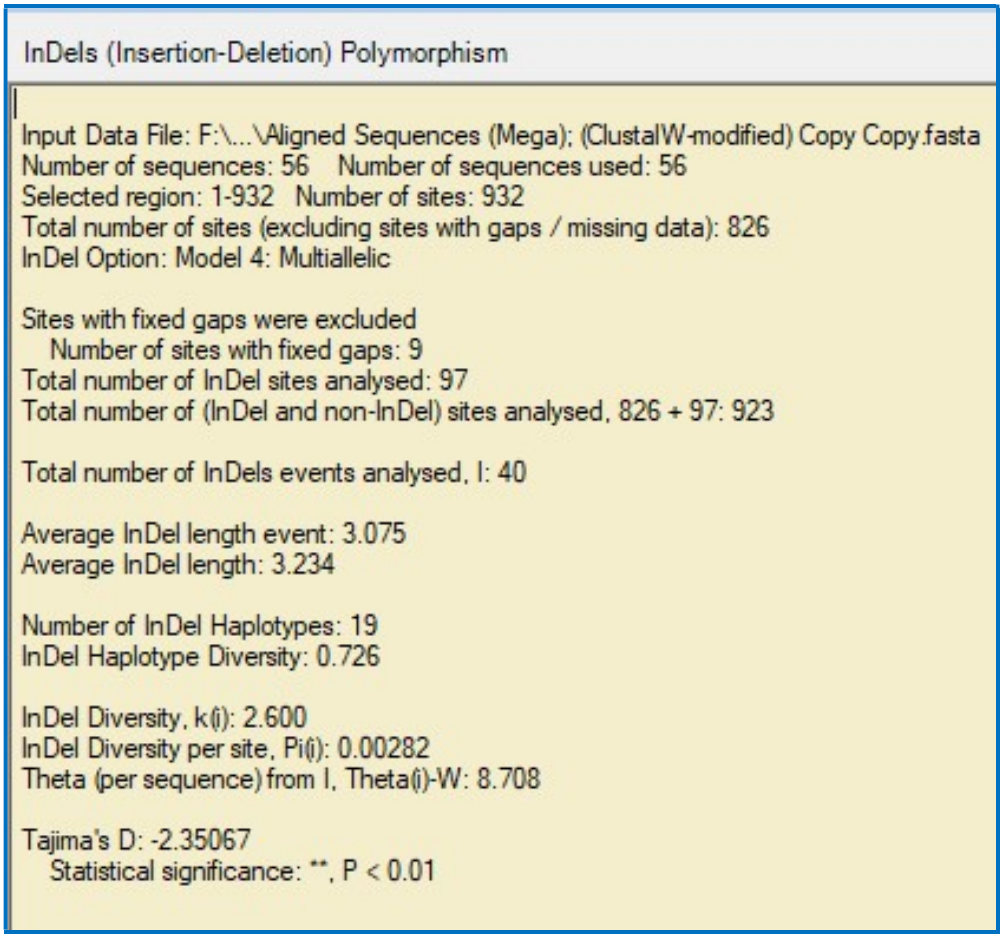

Figure 4.12: Showing the analysis of the InDel (Insertion-Deletion) Polymorphisms in the 932 bp region of mt. D-loop of Kenyan lion. Analysis done in (DnaSP Version 6.12.03)

The Northern frontier lions showed an InDel polymorphism of 52 sites and InDel diversity per site of 0.0086 while the Southern Frontier lions had 13 InDel sites and InDel diversity per site of 0.00201. Analysis of InDel site and InDel diversity among the six conservancies in the Maasai Mara ecosystem revealed that the Naboisho conservancy had the most InDel sites (28) and the highest InDel diversity (0.00318) while the lowest was Olare Motorogi and Olare

Naboisho with each 4 InDel sites and InDel diversity of 0.00139. Among the four prides studied, the Enesikiria pride showed 34 InDel sites and 0.0039 (In-Del) diversity per site followed by Engoyanai pride that showed 6 (In-Del) sites and 0.00201 (In-Del) diversity. However the Cheli pride had 2 (In-Del) sites and an (In-Del) diversity of 0.00039. The Moniko pride showed 4 In-Del sites and an In-Del diversity per site of 0.00116 (Table 4.3).

Table 4.6: Showing the number of InDel sites and InDel

Diversity per site per lion population

S/No.	Population	INDel Sites	InDel diversity
1	**Northern + Southern**	97	0.00282
2	**Northern Frontier**	52	0.00860
3	**Southern Frontier**	13	0.00201
4	**Maasai Mara NR**	12	0.00208
5	**Naboisho Conservancy**	28	0.00318
6	**Mara North Conservancy**	8	0.001156
7	**Olare Motorogi Conservancy**	4	0.00139
8	**Olare Naboisho Conservancy**	4	0.001395
9	**Ol Kinyei Conservancy**	5	0.00186
10	**Enesikiria Pride**	34	0.0039
11	**Cheli Pride**	2	0.00039
12	**Engoyanai Pride**	6	0.00201
13	**Moniko Pride**	4	0.00116

Haplotype analysis of the 932 bps region of the mitochondrial D loop of all the 56 individuals revealed 41 haplotypes and haplotype diversity of 0.964. There were four haplotypes that were shared by more than one individual lion clustering together. Haplotype 2 was the most

frequent haplotype which was shared by ten individual lions of which two individual lions were from Moniko pride (Lio 43, 45) and three lions from Engoyanai pride (Lio 39, 48, 118b). The others were two lions from Cheli pride (Lio 73, 86), one lion from Ol Kinyei conservancy (Lio 71), one lion from Ilkisiusiu pride (Lio 98) and one lion from the unknown location (Lio 116). Haplotype 11 was the second most frequent haplotype shared by four lions of which three lions were from Engoyanai pride (Lio 41, 51, 64) and one lion from Ol Kinyei conservancy (Lio 106). Haplotype 12 was the third most frequent haplotype shared by three lions of which one lion was from Maasai Mara National Reserve (Lio 103), one lion from Mara North coalition (Lio 104) and one individual lion from the offbeat coalition (Lio 105). Haplotype 36 was the fourth most frequent haplotype shared by two lions of which one lion was from Maralal (Lio 12) and another lion from Amboseli National Park (Lio 8B). All the other 37 haplotypes were not shared but had only one lion individual as a haplotype. Analysis of identity matrix indicated that individual lions in haplotypes 2 and 12 all had a 100% nucleotide identical with individuals in haplotypes 11 and 36 with slightly below 100% identity in their nucleotide composition. This was an indication that lions sharing similar haplotypes were very closely related (Figure 4.13-4.14).

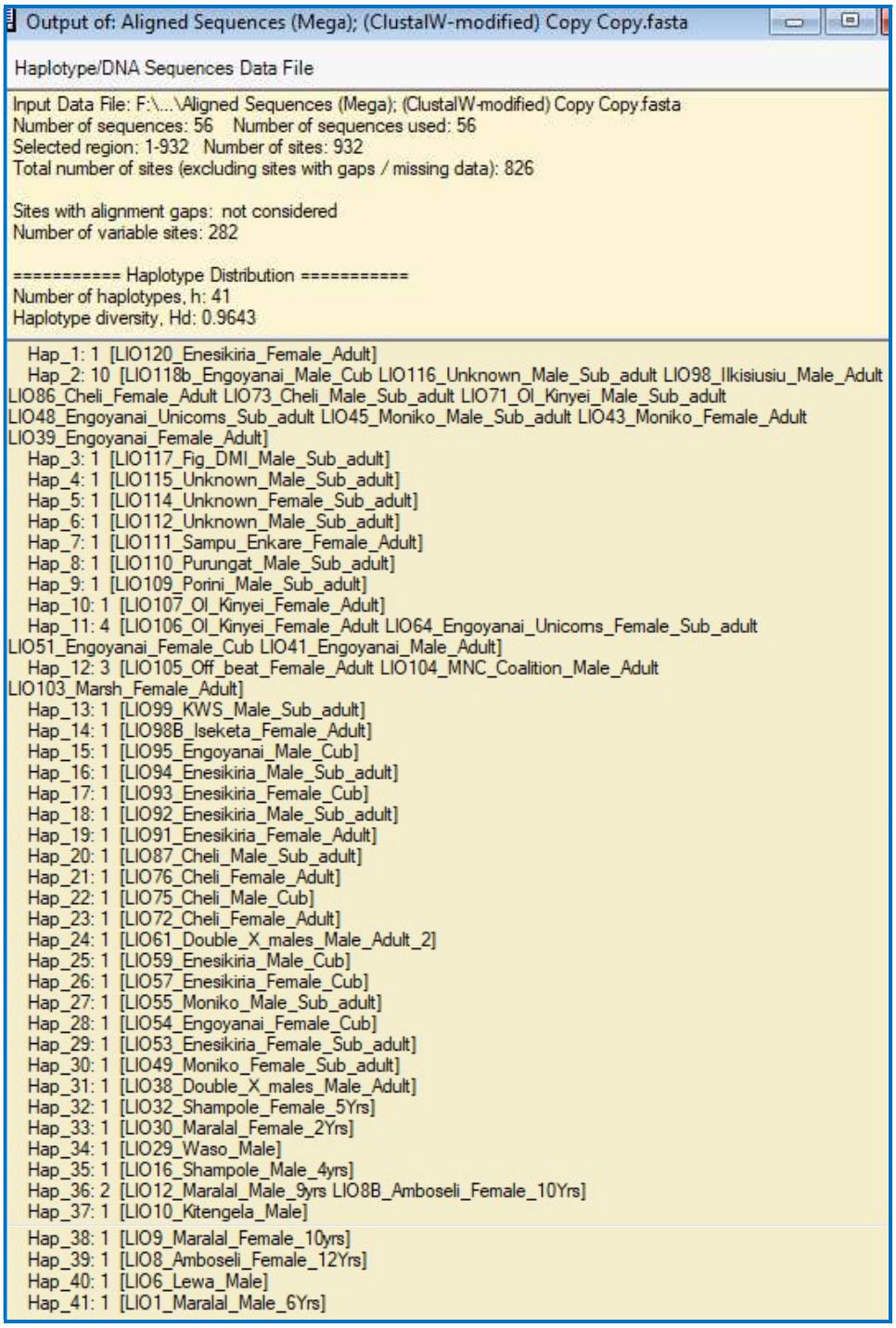

Figure 4.13: Showing 41 haplotypes and haplotype diversity in 932 bp mtDNA D-loop region of 56 DNA sequences of the of Kenyan lion populations. Analysis done in (DnaSP Version 6.12.03)

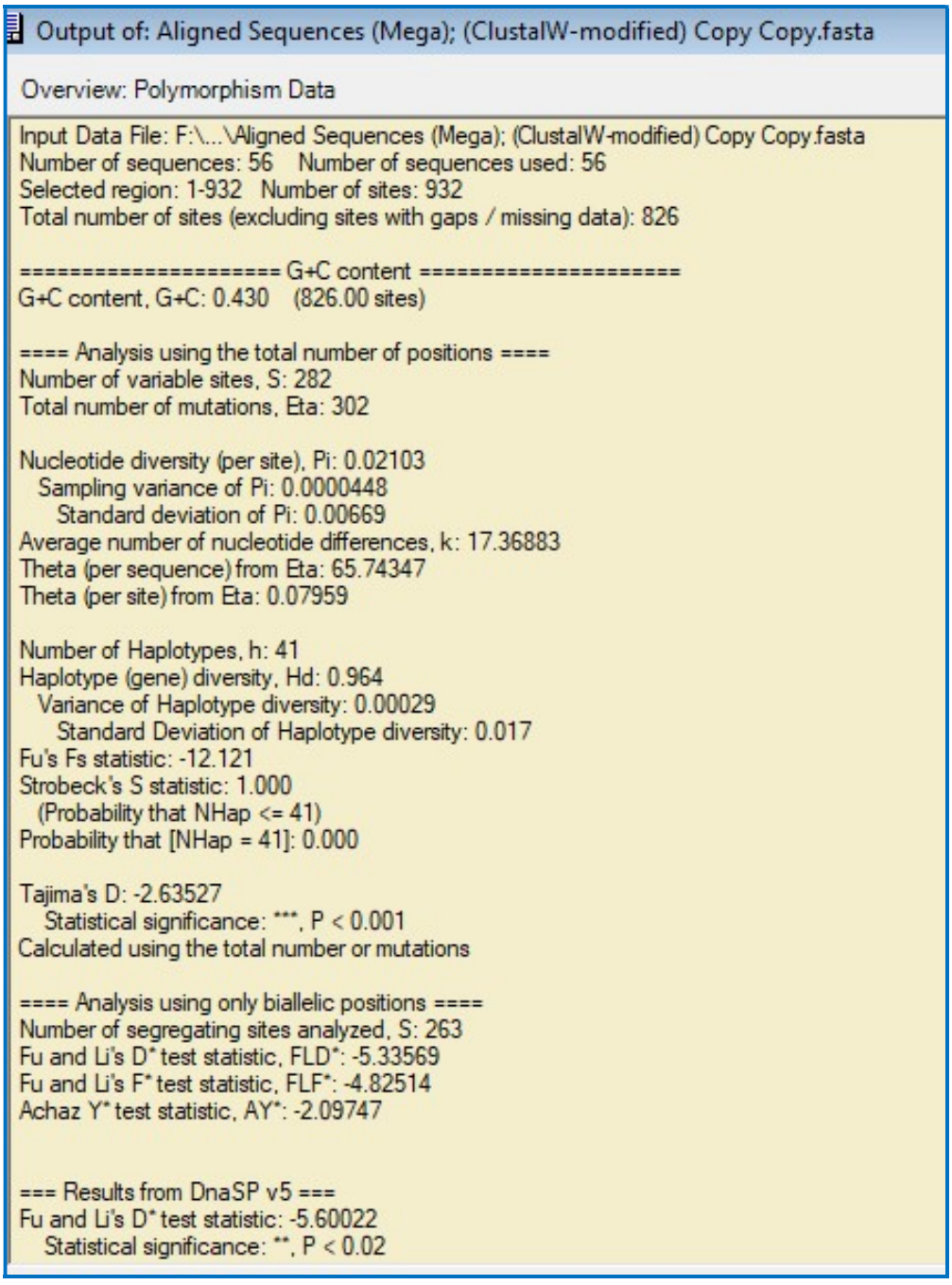

Figure 4.14: Showing the overview of polymorphism of the 932 bps region of mitochondrial D loop of Kenyan lion. Analysis done in (DnaSP Version 6.12.03)

4.7 Maternal lineages

The DnaSP version 6.03.12 software was used to analyze the data for the number of haplotypes and haplotype diversity. Analysis of haplotype distribution indicated that there were 41 haplotypes in the two populations with a high haplotype diversity (Hd) of 0.9643 and 4 haplotypes were shared (Figure 4.15). The lion individuals among the haplotypes 2, 11, 12 and 36 that were shared showed a percentage pairwise identity of 100% with each other and the lowest pairwise differences. This was an indication of the close relationships of the lions in each shared haplotype. The most frequent haplotype among the 41 haplotypes was haplotype 2 with 10 lion individuals all from the southern frontier population. This haplotype comprised of three lions from Engoyanai pride, two lions from Moniko pride, two lions from Cheli pride, one from Ol Kinyei, one from Iikisiusiu pride and one from unknown location. The second most frequent haplotype was haplotype 11 with four lion individuals also from the southern frontier. The four lions were three from Engoyanai pride and one from Ol Kinyei conservancy. The third most frequent haplotype was haplotype 12 with three lion individuals from the southern frontier. The three lions were one from Maasai Mara NR, one from Mara North and one from Offbeat pride. The fourth most frequent haplotype was haplotype 36 with two lions, one from the Amboseli NP in southern frontier and one from Maralal in the northern frontier. The other 37 haplotypes were not shared and were unique with one lion individual (Figure 4.15).

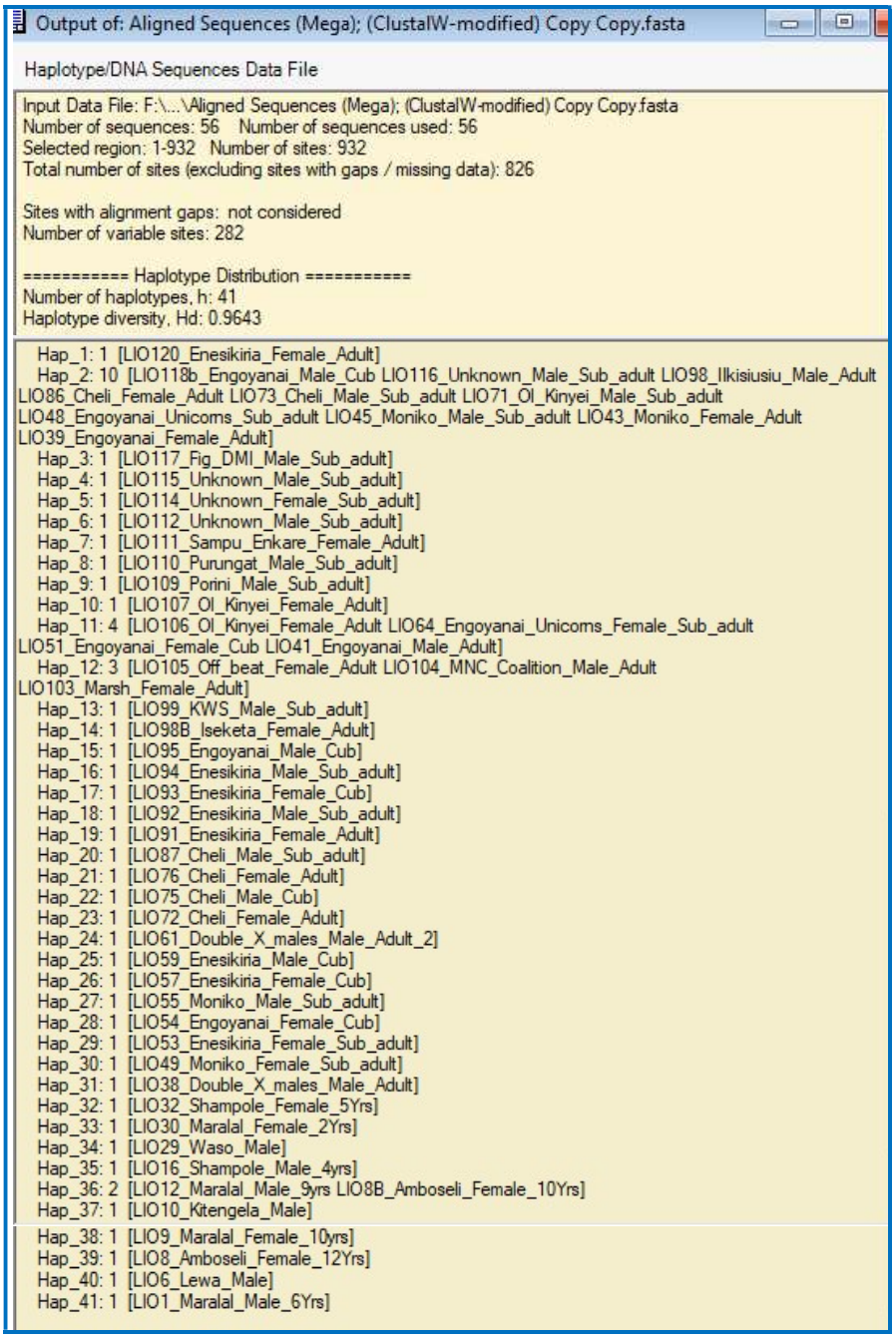

Figure 4.15: The number and distribution of 41 haplotypes of the northern and the southern lion populations analyzed in DnaSP version 6.03.12 (Shared Haplotypes 2, 11, 12 and 36, Haplotype 2:10 haplotypes, Haplotype 11:4 haplotypes, Haplotype 12:3 haplotypes, Haplotype 36:2 haplotypes)

Median joining network map (Figure 4.16) was constructed in PopArt version 1.7 software to show the lion populations historical events and to demonstrate their haplotype relationships. This indicated a close relationship between the number of nucleotide substitutions (mutations) from the possible ancestral state and the pairwise distances, percentage pairwise similarity, percentage pairwise differences. The greater the pairwise distance from the possible ancestral state of each individual haplotype, the lower the percentage pairwise similarity, the greater the percentage pairwise differences and the higher the number of mutations and vice versa (Figure 4.16). Lions from the northern frontier showed the largest pairwise distance, the lowest percentage pairwise similarity, the highest percentage pairwise differences and the highest number of mutations. For example, LIO 6 (Lewa) showed percentage pairwise similarity of 81.5%-82.9%, pairwise differences of 0.154-0.191 and 128 mutations being the highest, LIO 1 (Maralal) showed pairwise similarity of 84.6%-86.24%, pairwise differences of 0.115-0.257 and 93 mutations, LIO 29 (Waso) showed pairwise similarity of 93.8%-95.14%, pairwise differences of 0.006-0.086 and 22 mutations and LIO 30 (Maralal) showed pairwise similarity of 94.2%-95.4%, pairwise differences of 0.027-0.041 and 18 mutations all from the northern frontier. An exceptional high level of mutations (50 mutations) was seen in an individual haplotype from Enesikiria pride (Lio 93) which is from the southern frontier (Figure 4.16).

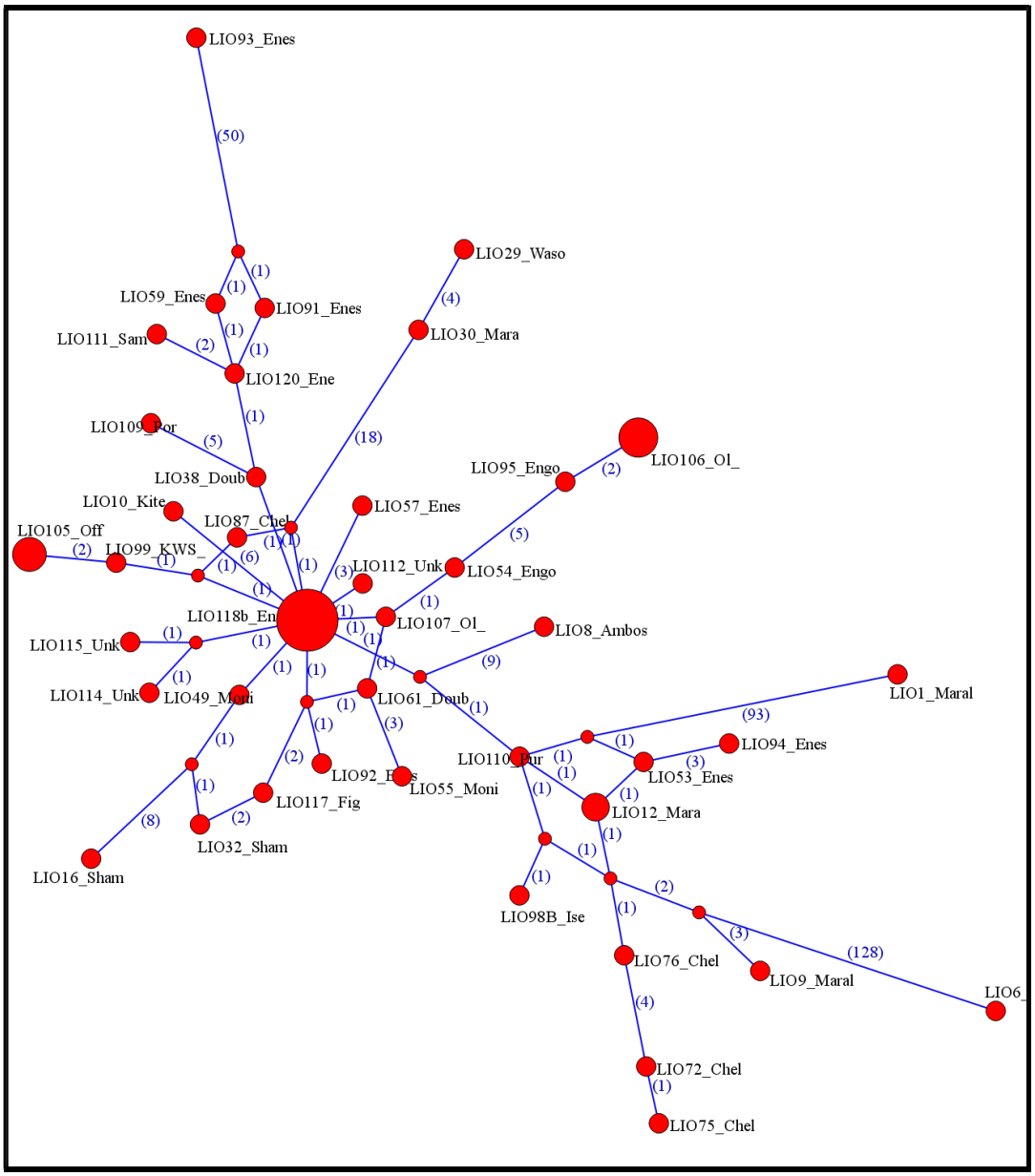

Figure 4.16: Median Joining Network Map showing relationship between the northern and southern lion population in Kenya. The area of circle correspond to the haplotype frequency, length of the lines corresponds to distance between the haplotypes, numbers in parenthesis correspond to the number of mutations or substitutions between the haplotypes, where the lines cross each other without a circle corresponds to possibility of an ancestral state

The evolutionary history and relationships of the lion population in the northern and the southern frontier was inferred in phylogenetic trees drawn in MEGA X software to show their maternal lineages. Three nucleotide substitution models were used including Kimura-2 Parameter model, Tamura-Nei and Hashegawe-Kishino-Yano substitution models. Original phylogenetic Trees were obtained from the highest log likelihood values for each substitution model showing branch lengths which were inferred as the number of substitutions per site in MEGA X. In Kimura-2 parameter model, Trees were obtained at the log likelihood value of -3094.19 (Figure 4.17-4.18), in Tamura-Nei parameter model, trees were obtained at log likelihood value of -3053.71 (Figure 4.20-4.21) and in HKY, trees were obtained at log likelihood value of -3054.03 (Figure 4.23-4.24). Original phylogenetic Trees for the three nucleotide substitution models showed a close relationships between the genetic distances of haplotypes Lio 06, Lio 93, Lio1 and Lio 29 which was the number of nucleotide substitutions per site experienced by these lion haplotypes. Bootstrap Consensus phylogenetic trees were also obtained and sub-trees with branches supported by strong bootstrap values of 50% or higher were shown as distinct haplogroups. Sub trees with branches supported by less than 50% bootstrap support values were collapsed because their haplogroups were unresolved. The relationships of these haplotypes in this haplogroup could not be determined or assigned to any distinct haplogroup (Figure 4.19, 4.22 and 4.25).

The rooting of the Maximum likelihood consensus phylogenetic trees with the three out groups, the leopard (*panthera pardus*), jaguar (*panthera onca*) and the tiger (*panthera tigris*) using the three nucleotide substitution models sorted the northern and the southern lion populations in to a single monophyletic clade, one maternal lineage and six haplogroups (A-F) (Figure 4.19, 4.22 and 4.25). The three nucleotide substitution models resolved the

phylogenetic relationship of the 56 lion individuals in Kenya into a single maternal lineage. The three consensus phylogenetic trees of all the substitution models also resolved the two subpopulations into five unique and distinct haplogroups (A-E) and another sixth unresolved haplogroup (F1-F5). The distinct haplogroups were supported by bootstrap value equal to or greater than 50%. The haplotypes of lion individuals in the same haplogroup was an indication of their close relatedness than haplotypes in different haplogroups (Figure 4.19, 4.22 and 4.25). Although the haplotypes in haplogroup F (F1-F5) had shown some relationships with the distinct haplogroups in the original Trees, they were supported by bootstrap value less than 50% and were collapsed during the analysis. These haplotypes were distantly related compared to other haplotypes in the haplogroups A-E. Their relationships depended on the bootstrap support. The smaller the bootstrap support, the far distantly related they are.

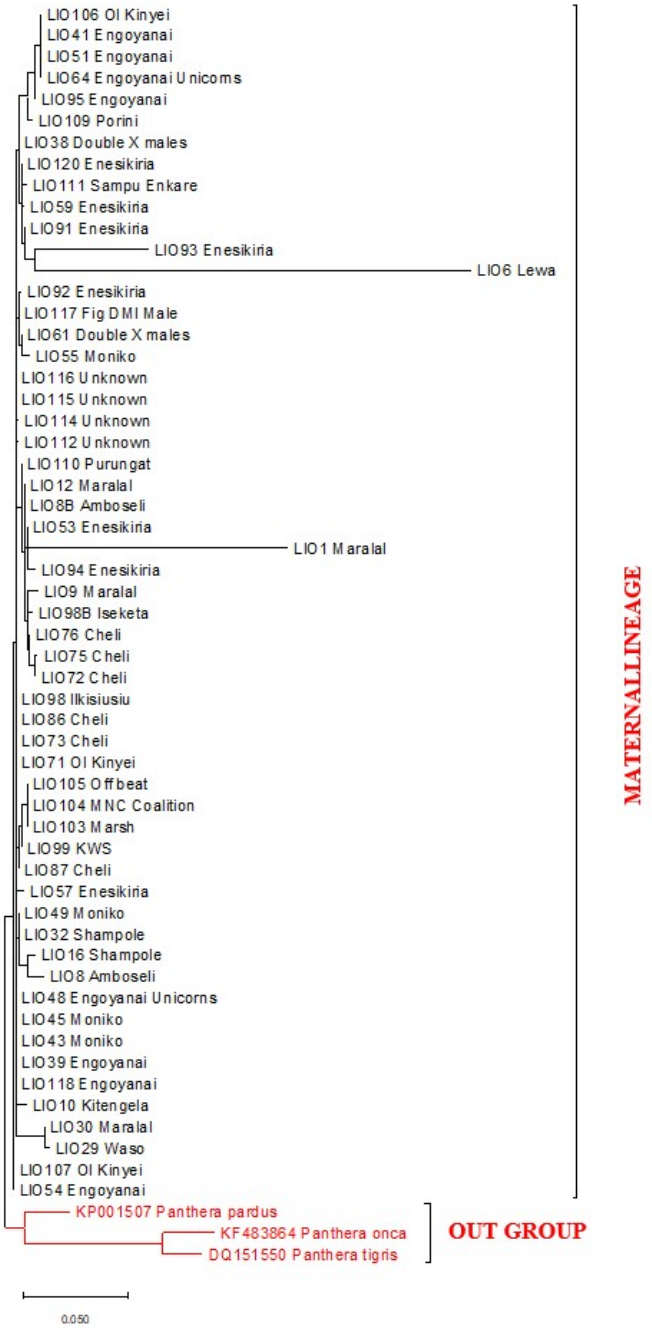

Figure 4.17: Original Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Kimura-2 Parameter Substitution Model. The Tree was rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and drawn to scale with Branch lengths measured in the Number of Substitutions per Site. (Drawn with MEGA X software)

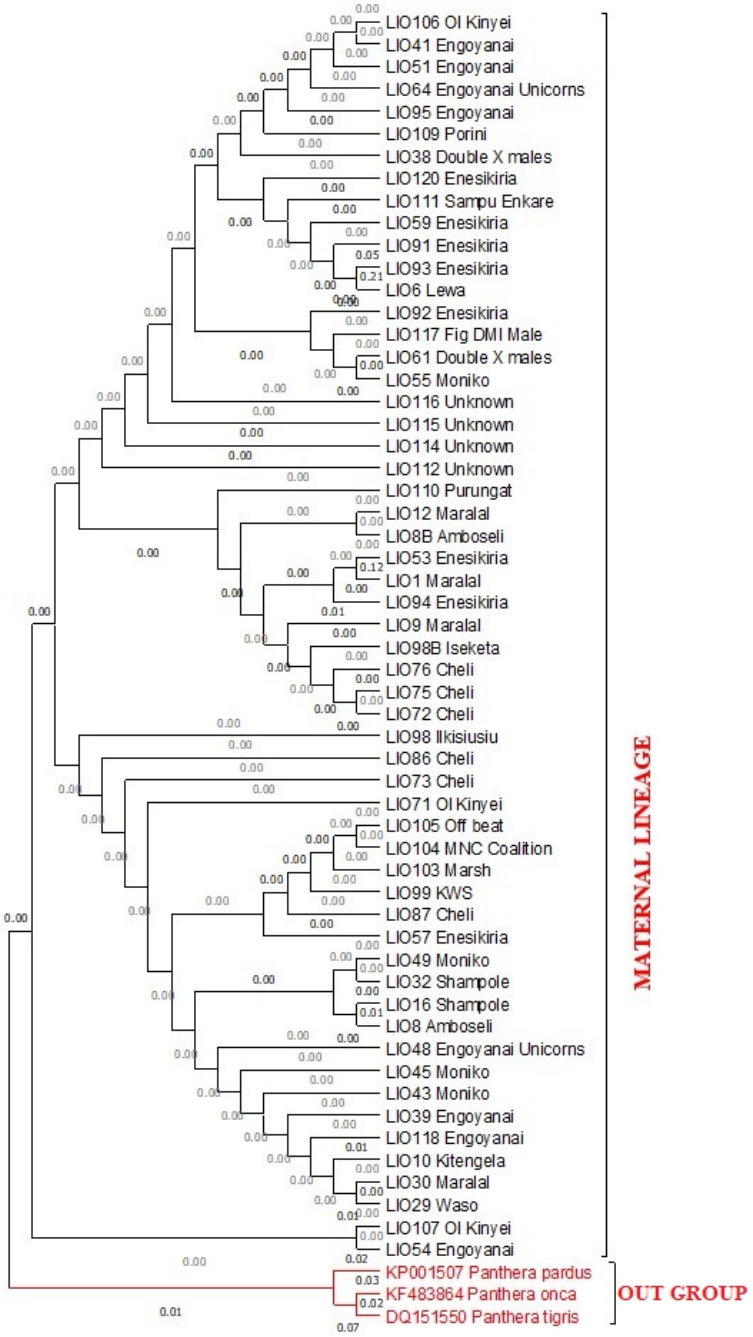

Figure 4.18: Original scaled Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Kimura-2 Parameter Substitution Model. The Tree is rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and the numbers correspond to bootstrap support values in %. The Letters labled A to F indicates Haplogroups (Drawn with MEGA X software).

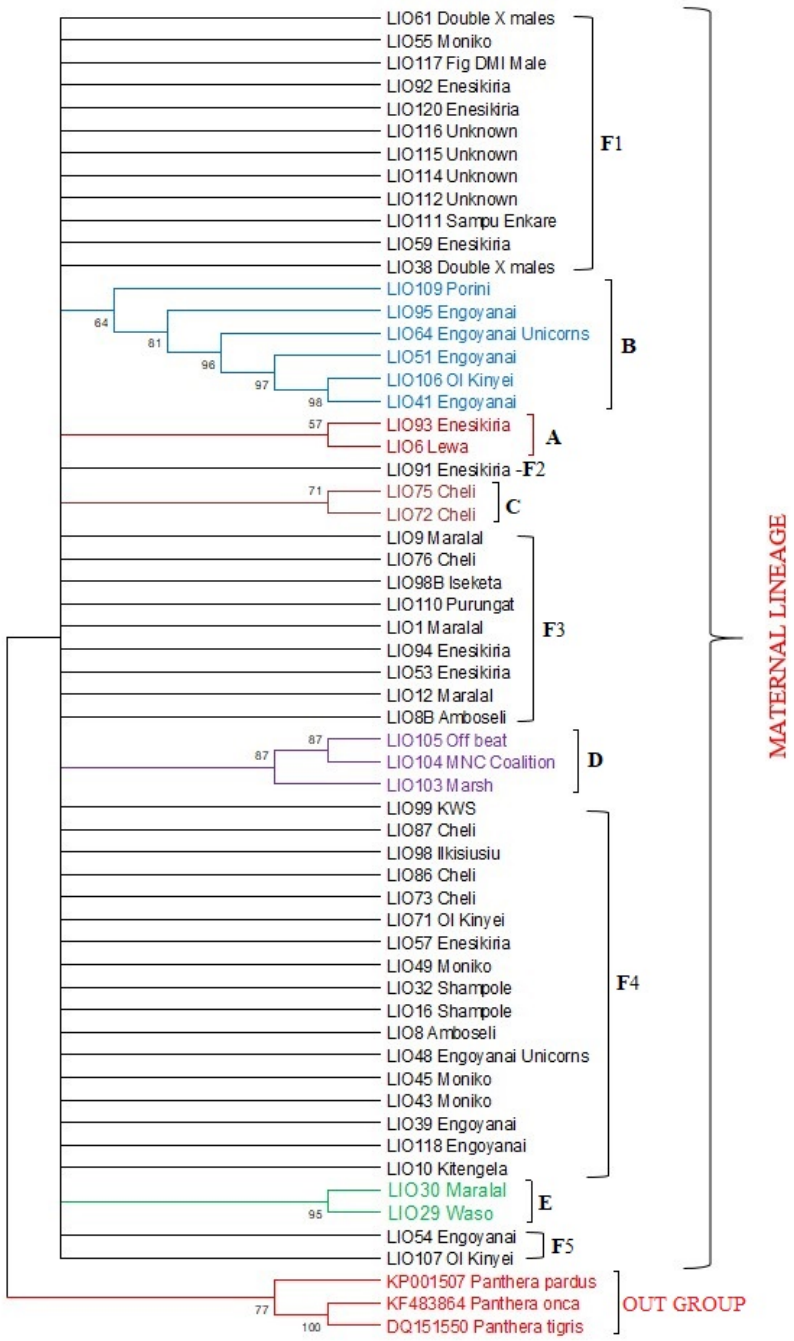

Figure 4.19: Bootstrap Consensus Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Kimura-2 Parameter Substitution Model. The Tree is rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and the numbers correspond to bootstrap support values in %. The Letters labled A to F indicates Haplogroups (Drawn with MEGA X software).

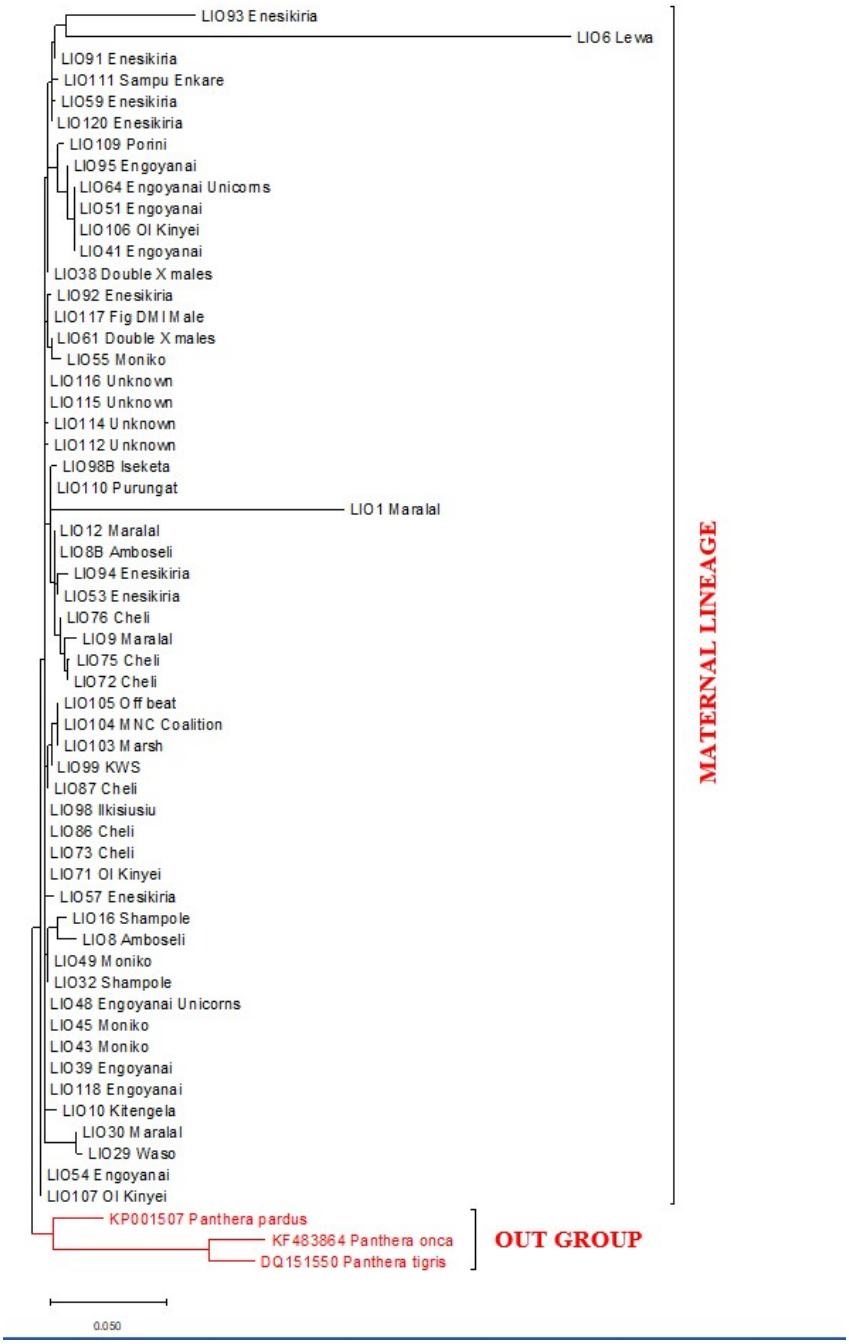

Figure 4.20: Original Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Tamura-Nei Substitution Model. The Tree was rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and drawn to scale with Branch lengths measured in the Number of Substitutions per Site. (Drawn with MEGA X software)

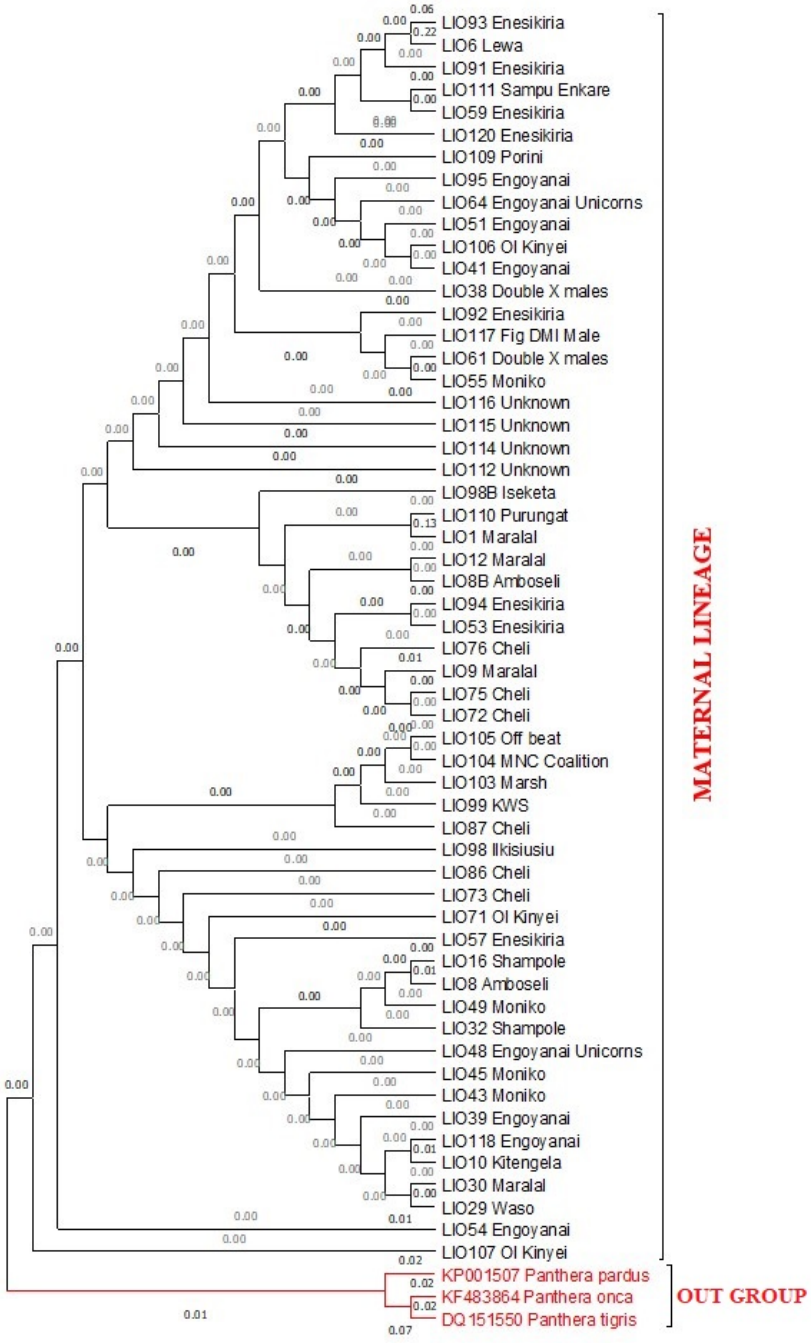

Figure 4.21: Original scaled Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Tamura-Nei Substitution Model. The Tree was rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and drawn to scale with Branch lengths measured in the Number of Substitutions per Site. (Drawn with MEGA X software)

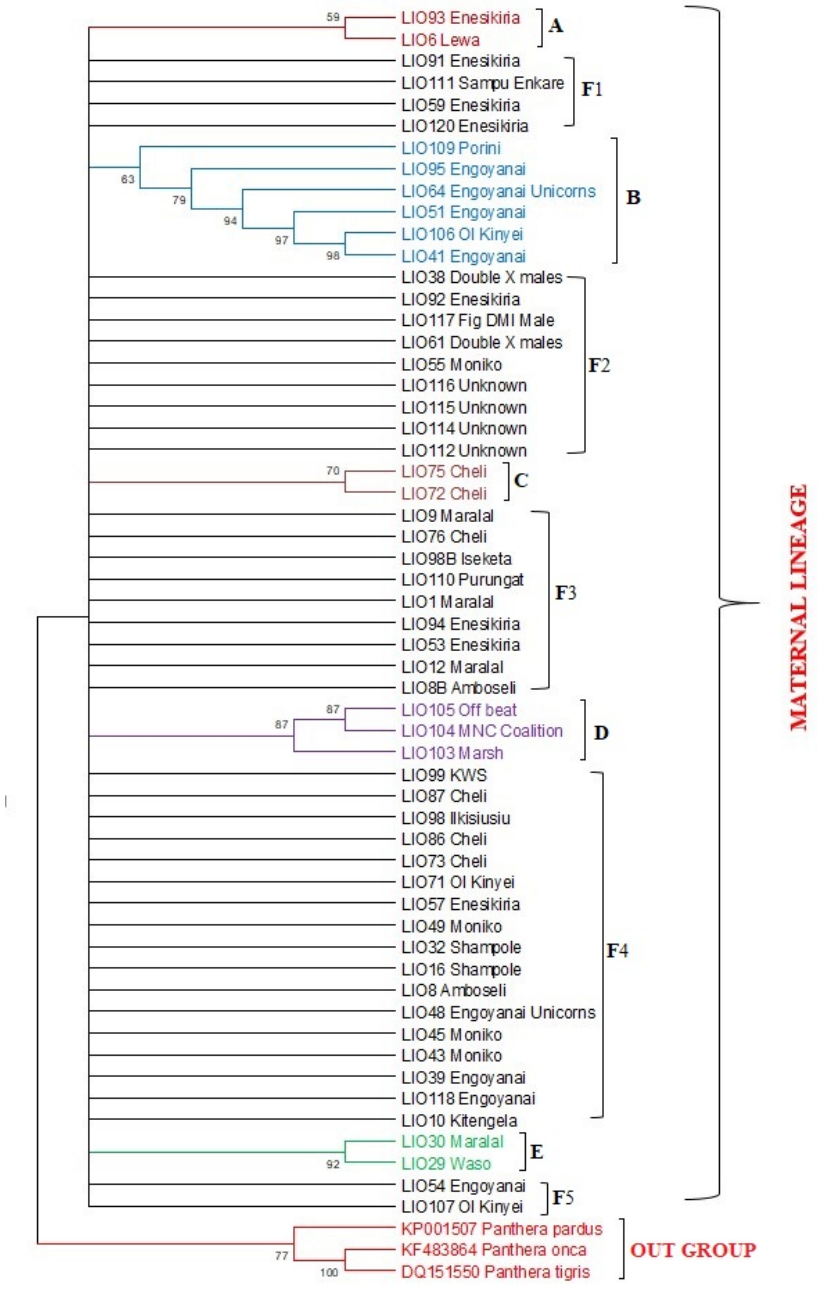

Figure 4.22: Bootstrap Consensus Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Tamura-Nei Substitution Model. The Tree is rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and the numbers correspond to bootstrap support values in %. The Letters labled A to F indicates Haplogroups (Drawn with MEGA X software).

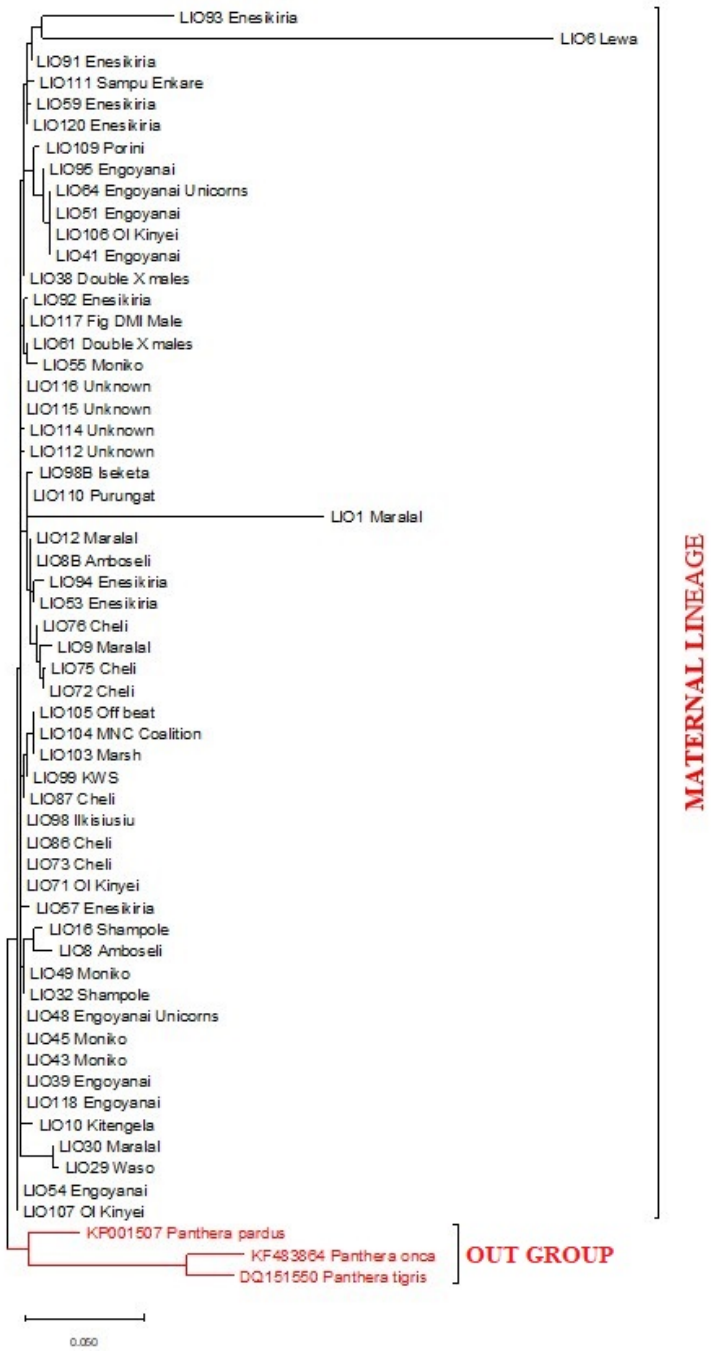

Figure 4.23: Original Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Hashegawe-Kishino-Yano (HKY) Substitution Model. The Tree was rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and drawn to scale with Branch lengths measured in the Number of Substitutions per Site. (Drawn with MEGA X software)

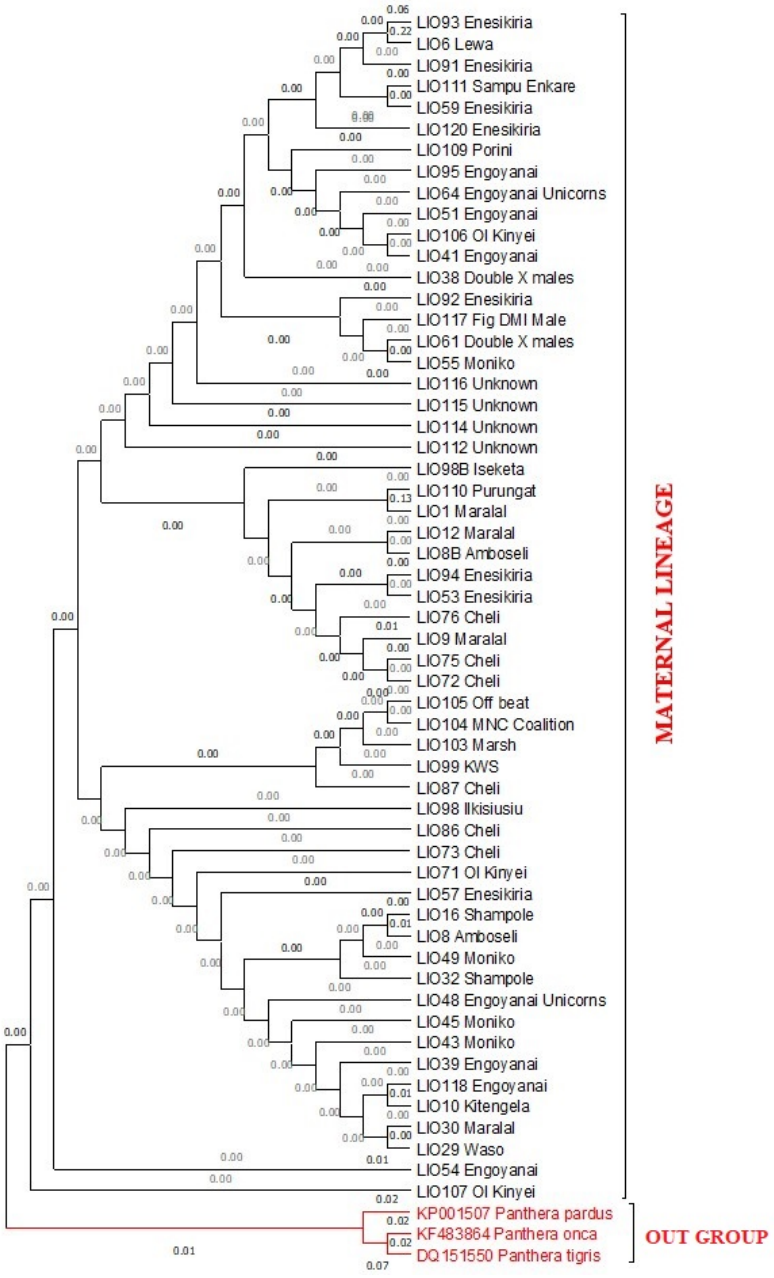

Figure 4.24: Original scaled Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Hashegawe-Kishino-Yano (HKY) Substitution Model. The Tree was rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and drawn to scale with Branch lengths measured in the Number of Substitutions per Site. (Drawn with MEGA X software)

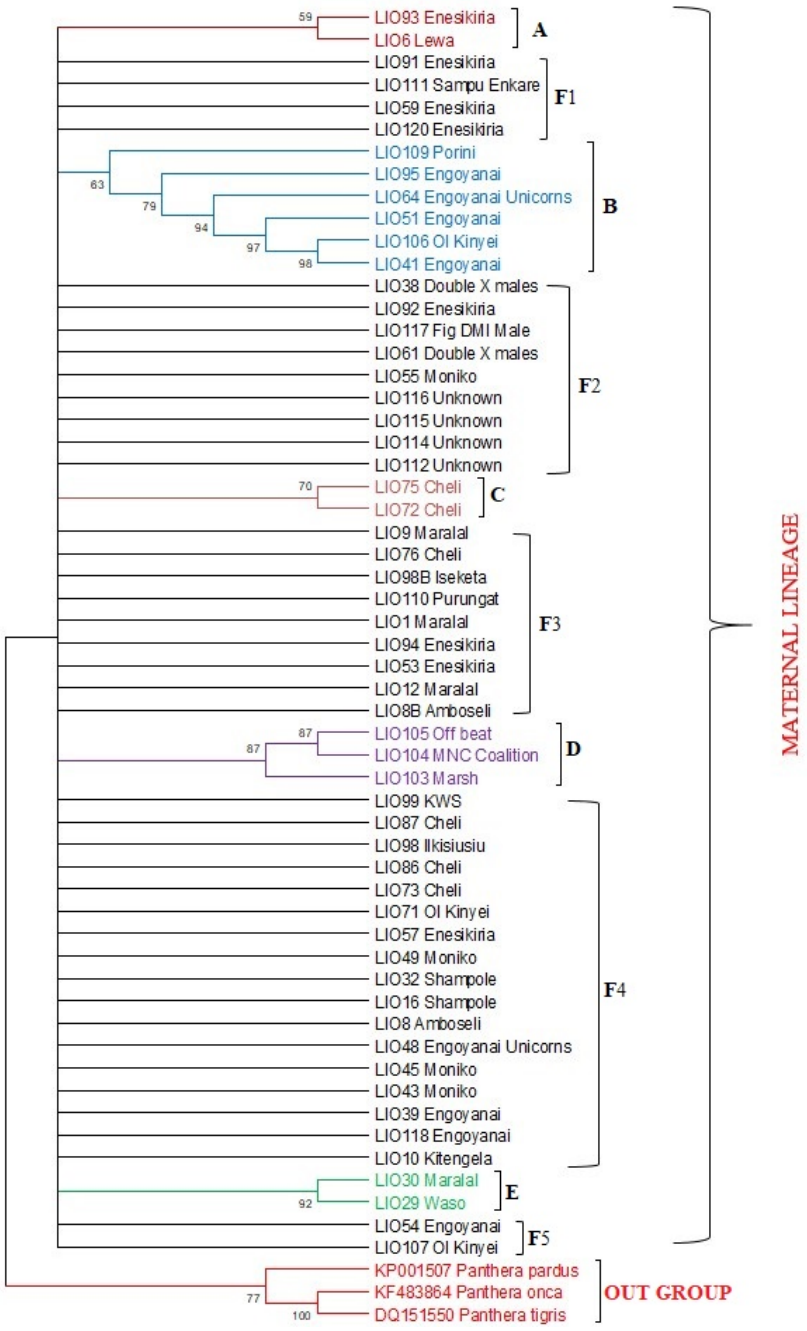

Figure 4.25: Bootstrap Consensus Phylogenetic Tree to infer evolutionary history using Maximum Likelihood method and Hashegawe-Kishino-Yano (HKY) Substitution Model. The Tree is rooted by three out groups, *Panthera pardus, Panthera onca* and *Panthera tigris* and the numbers correspond to bootstrap support values in %. The Letters labled A to F indicates Haplogroups (Drawn with MEGA X software).

CHAPTER FIVE

5.0 DISCUSSION

Panthera leo melanochaita, one of the most important Kenya's big five wildlife animals is the country's main flagship species (Caro, 2010). It boosts the country's economic growth through international and local tourism, trophies and conservation of biodiversity (Lindsey et al., 2007a; Caro and Riggio, 2013, 2014). Their history has been marked by a worrying trend of decrease in their population (Craigie et al., 2010). The most recent IUCN assessment of the African lion's trends over three lion generations showed a decline of 43 % (IUCN, 2018). A few stable populations in four southern African lion populations including South Africa, Botswana, Zimbabwe and Namibia reported 12% increase and a decline in the rest of Africa including Kenya (Bauer et al., 2016; IUCN, 2018).

Majority of the Kenyan people depend on agriculture for their livelihoods; because of the size of the fast growing human population, most agricultural practices have been extended to wildlife conservation and dispersal areas. This has left the formally interconnected *Panthera leo melanochaita* populations in Kenya in fragmented states due to reduced lion prey base, loss of natural habitats and human wildlife conflicts (IUCN, 2018). Because of this, conservation intervention measures are warranted. The mitochondrial genome especially the D-loop, cytochrome *b*, 12S and 16S rRNAs (Lorenzen et al., 2012) have recently been used to show genetic diversities, variations and phylogeographic delineation of African lions into different clades based on their geographical locations (Barnett et al., 2009; Charruau et al., 2011; Bertola et al., 2016). The mitochondrial D-loop (130 bps) indicated six phylogeographic groups of the African lions with a north-south taxonomic divisions (Barnett et al. 2006 b, 2014; Bertola et al. 2016). Revealing the genetic structure of *Panthera leo melanochaita*

populations in Kenya using the mitochondrial D-loop variations in genetic polymorphisms, diversity and evolutionary relationships in this study may help in conservation intervention.

In the present study, the 932 bp mitochondrial D-loop was analyzed to measure and estimate genetic variations in polymorphism, diversity and evolutionary relationships of lions in the northern and southern frontiers in Kenya. This could help explain the hypothesis that the northern and the southern frontier lion populations in Kenya are genetically similar with low genetic diversity. The population presented mitochondrial D loop polymorphism with 282 variable (polymorphic) sites characterized by 211 singleton variable sites (s) and 71 parsimony informative sites (pi). Singleton variable sites of two, three and four nucleotide variants and parsimony informative sites of two and three nucleotide variants characterized the northern and the southern frontier populations. However, individual sub populations displayed only two and three singleton variable and parsimony informative sites. The northern frontier population and the Enesikiria pride recorded higher levels of polymorphism than all other the sub-populations (Table 4.1). The trends seen in the population genetic polymorphism corresponded with the levels of mean genetic diversity, mean genetic distances, polymorphic sites, nucleotide diversity and average nucleotide differences that were observed in the entire population and sub populations.

The entire lion population exhibited low levels of mean genetic diversity (0.04 to 0.052), low nucleotide diversity (Pi=0.0213) but high haplotype diversity of (Hd=0.964). This could be due to the small population sizes that could have undergone differentiation due to multiple underlying factors affecting the lion population. The northern frontier lions presented high levels of polymorphism and diversity compared to other populations while the lions in the prides presented the lowest levels of both mean genetic diversity (0.01) and nucleotide

diversity (0.00312 to 0.02). However the Enesikiria pride had an exceptional higher levels than all the other prides and individual conservancies in the southern frontier. The northern frontier lions, southern frontier lions and individual conservancies displayed very close levels of between group mean genetic distance (0.04-0.0504) and mean genetic diversity (0.04 to 0.052). This could be an indication that they could be genetically closely related.

The geographical location could also have played a key role in influencing the genetic distances and genetic diversity between populations (Liu et al. 2019). The geographical location of the northern frontier lion population could be the reason for their higher levels of within group mean genetic distance (0.08) and nucleotide diversity (pi=0.082) than the within group mean genetic distance (0.012) and nucleotide diversity (pi=0.0113) seen in the southern frontier counterparts. Closely related individuals exhibit less genetic variations as observed in the southern frontier lions with smaller within group mean genetic distance (0.012) relative to the northern frontier lions (0.08). Lions in the northern frontier exhibited high level of genetic polymorphisms and diversity than the southern frontier lions. The patterns of dispersal also influence how genetic diversity is distributed in lion populations in lions (Tende et al., 2014a, b). Although the population in the northern frontier could be small, lack of geographic barrier or few artificial barriers to gene flow could give the population an advantage to depict the observed higher levels of polymorphism and diversity as opposed to the southern frontier populations. The geographical distance may also influence the between group mean genetic distances and nucleotide divergence between populations. The between group mean genetic distances between the northern frontier conservancy and individual conservancies in the southern frontier were higher compared to the nucleotide divergence observed between the lions in the prides.

The northern part of Kenya covering the areas of Marsabit, Maralal, Waso, Isiolo, Moyale and Garissa is basically Arid and Semi-Arid lands (ASALs) with low rainfall patterns around the year (Ojwang' et al., 2017). The area has very few lions (IUCN, 2018) and because agricultural and anthropogenic activities in this frontier are rare with low human population density; lions have wide geographic ranges and dispersal corridors as compared to the southern frontier areas of Maasai Mara ecosystem. Lack of known geographical barriers and probably few artificial barriers such as roads and infrastructural developments in the area led to low interrupted gene flow and minimal population fragmentation relative to the southern frontier population. The Maasai Mara ecosystem in south western Kenya experiences high levels of bi-modal annual rainfall patterns (Ojwang' et al. 2017). The area is also highly populated with numerous human activities including intensive farming and pastoralism which normally commercialize on livestock farming. This has highly put a lot of pressure on wildlife conservation due to farming activities being entrenched in to conservation areas leading to human-wildlife conflicts and lion killing by the communities (van Eeden et al., 2018; Ontiri et al., 2019). This in addition to several other threats mentioned in this study has resulted to loss of numerous lions by spearing and poisoning (Frank et al.2008; van Eeden et al., 2018; Ontiri et al., 2019) leading to reported decrease of lions in Kenya (Frank et al.2008; IUCN, 2018). The anthropogenic and agricultural activities in this area play a major role as artificial barriers and points of habitat disconnection for lions and other wildlife animals. As indicated earlier, individuals showing smaller within group mean genetic distances are closely related and have less genetic variations as is the case exhibited by individual conservancies (0.01) in the southern frontier (Table 4.4).

The entire lion population was characterized by low nucleotide diversity (Pi=0.0217). Among

the conservancies in the southern frontier, lions in the private conservancies showed high levels of nucleotide diversity than lions in the Maasai Mara NR. The levels of nucleotide diversity ranged from 0.0062 to 0.00896 with the lions in Mara North having the highest diversity (pi=0.00896) and the Maasai Mara NR having the lowest (pi=0.0055). This could suggest that lions in the privately owned conservancies are well protected than state owned conservancies. It could further suggest that the wildlife around in the Maasai Mara state owned land could be facing more human population pressure due to land use changes than privately conserved populations. The low genetic polymorphism and diversity observed in most sub-populations could be attributed to genetic processes and the fragmented state of the study population caused by underlying threats and require genetic rescue.

The levels of differentiation are measured by fixation index and range between (F_{st} =0 and 0.25) in mammals (Meirmans and Hedrick, 2011). The southern frontier population exhibited slightly higher levels of genetic differentiation (F_{st} =0.069-0.165) (Table 4.6) relative to the northern frontier lion population (F_{st} =0.066). Although this data is not sufficient to fully evaluate the levels of genetic differentiation in this study, it could serve as an indication of early signs of genetic processes in these conservancies.

Lions are the most social felids and live in families called prides; each pride consist of a total of 2-35 lions, with 2-18 related females, their cubs and a coalition of 1-9 males (Sogbohossou et al., 2014; Verschueren, 2017). The genetic diversity of lion population is adversely reduced in situations where lions experience extra threats to anthropogenic killing like; trophy hunting of females and young lions, pride take over by new males which lead to infanticide of cubs in prides (IUCN, 2018). Additionally uncontrolled hunting of males below recommended seven years and over and failure to adhere to other trophy hunting regulations like hunting one male

lion per 2000 km^2 results to disruption of lion's social structures and reduction of genetic diversity (Loveridge et al., 2007, 2010; Mweetwa et al. 2018; IUCN, 2018). Lions in the four prides studied showed smaller within group mean genetic distances (0.00-0.025) indicating that they were very closely related to each other with low levels of genetic polymorphisms. The lions in these prides further exhibited low levels of within sub-population mean genetic diversity (0.01-0.015) and low mean genetic diversity (0.01-0.02) in entire population of each two prides. They also exhibited moderate levels of genetic differentiation (F_{st}=0.0617-0.34) (Table 4.6) which is a sign of gene flow barrier.

The levels of differentiation exhibited by lions in the four prides were closely similar to the levels of differentiation showed by lions in the two conservation units in Nigeria with differentiation level reaching to (Fi_s=0.21) between 2008 and 2012. The two lion CUs included the Kainji lake National Park (KLNP) which also showed (Fi_s=0.38) and the Yankari Game Reserve with (Fi_s=0.49) between 2009 and 2012 (Tende et al., 2014a). The Namibia's Etosha National Park similarly showed high levels of inbreeding (Fi_s=0.37) (Antunes et al., 2008; Tende et al., 2014a). Similarly, low levels of gene flow and differentiation were also seen in mountain lions of California and were suggested to have resulted from anthropogenic obstacles (Riley et al., 2006). Other species also demonstrated similar levels including the Scandinavian wolf (*Canis lupus*) with (Fi_s=0.41) (Tende et al., 2014a; Laikre et al., 2018) and brown bear (*Ursus arctos*) with (Fi_s=0.37) (Tende et al., 2014a; Akesson et al., 2016). This could also suggest that the observed level of genetic polymorphisms, diversities and variations of lions in Kenya is likely to be observed even in other species in Kenya.

The phylogenetic relationship demonstrated by the mitochondrial D-loop among *Panthera leo melanochaita* fragmented populations in Kenya with the three out-groups of *Panthera species*,

Panthera onca, *Panthera tigris* and *Panthera pardus* indicated that all *Panthera* genus have a close relationship and a common ancestor. This is in agreement with the previous study of the complete mitochondrial D-loop of *Panthera leo*, *Panthera tigris*, *Panthera uncia* (snow leopard), *Panthera onca* and *Panthera pardus* done to compare their mitochondrial D-loop genetic variations and structural features. The study established that the five species diverged from a common ancestor about three to four million years ago (Jae-Heup et al. 2001). The three *Panthera genus* and the studied lion population formed a single monophyletic group with a common ancestor and a single maternal lineage. This was an indication that the studied lion population diverged from one maternal line and with time due to genetic alterations by nucleotide substitutions, resulted to other distinct haplogroups. The occurrences experienced in their genetic structure through nucleotide substitutions or mutations over time resulted to the 41 haplotypes which further diverged to the five distinct haplogroups and the sixth haplogroup that had not yet been resolved. This study serve as evident that few genetic changes have been experienced in this study population as only few haplotypes have been resolved into five distinct haplogroups by the three nucleotide substitution models. However, more specific studies with other molecular markers such as microsatellites are needed to unravel this uncertainty.

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

- This study found out that the 932 bp mitochondrial D loop region of the 56 lion population exhibited 282 variable (polymorphic) sites with 211 singleton variable sites of two, three and four nucleotide variants and 71 parsimony informative sites of two and three nucleotide variants. The northern frontier lion population exhibited higher levels of polymorphism than the southern frontier lions, individual conservancies in the south and prides.
- This study also found out that the northern frontier and the southern frontier lion populations in Kenya exhibited low levels of mean genetic diversities (0.04 to 0.05) within sub-population and entire population and nucleotide diversities (pi=0.02103). There was a close relationship observed between within group mean genetic distance and nucleotide diversity. The higher the within group mean genetic distance, the higher the nucleotide diversity and vice versa. The northern frontier lion population exhibited higher level of nucleotide diversity than the southern frontier lions. However, the two populations exhibited significant haplotype diversity (Hd= 0.964).
- Phylogenetic analysis showed a single monophyletic clade, one maternal lineage and six haplogroups. Five haplogroups (A-F) were distinct as revealed by the inference of phylogenetic trees rooted with three closely related *Panthera* species and three nucleotide substitution models in this study. Other haplotypes in one haplogroup (F) were not yet been resolved in to their distinct haplogroups.

6.2 Recommendations

- This study recommends that more research studies to be carried out using other genetic markers including microsatellite markers, other mitochondrial DNA regions and nuclear genetic markers.
- The study revealed low levels of mean genetic diversity and recommends an urgent action for genetic rescue for the northern and the southern frontier lion populations to recover the lost genetic integrity in Kenya. The southern frontier lion population should be given special attention for its low polymorphism and genetic diversity compounded by the underlying threats.
- The deteriorating nature of the population size and genetic structure in terms of polymorphism and mean genetic diversity in the current extant lion population in Kenya as a result of declining trends and underlying threats may completely be lost if conservation measures are not considered immediately.
- Regular surveys and monitoring of the lion population should be conducted to establish the exact numbers or estimates in the lion conservation units in Kenya.

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APPENDICES

Appendix I: List of samples sequenced in the study

S/NO	LION ID	SEX	AGE	LOCATION/PRIDE	HOMERANGE
1	LIO1	Male	6	Maralal	Northern Frontier
2	LIO6	Male	-	Lewa	Northern Frontier
3	LIO8	Male	Ten	Amboseli	Amboseli
4	LIO8B	Male	Ten	Amboseli	Amboseli
5	LIO9	Female	1	Maralal	Northern Frontier
6	LIO10	Male	-	Kitengela	Nairobi National Park
7	LIO12	Male	9	Maralal	Northern Frontier
8	LIO16	Male	4	Shampole	Soralo/Magadi
9	LIO29	Male	-	Waso	Northern Frontier
10	LIO30	Female	2	Maralal	Northern Frontier
11	LIO32	Female	5	Shampole	Soralo/Magadi
12	LIO36	Male	7	Maralal	Northern Frontier
13	LIO38	Male	Adult	Double X Males	Maasai Mara National Reserve
14	LIO39	Female	Adult	Engoyanai	Olare Motorogi conservancy
15	LIO40	Male	Cub	Engoyanai	Olare Motorogi conservancy
16	LIO41	Male	Cub	Engoyanai	Olare Motorogi conservancy
17	LIO43	Female	Adult	Moniko	Olare Motorogi conservancy/Naboisho conservancy
18	LIO45	Male	Sub adult	Moniko	Olare Motorogi Conservancy /Naboisho Conservancy
19	LIO48	Female	Sub adult	Engoyanai Unicorns	Olare Motorogi conservancy
20	LIO49	Female	Sub adult	Moniko	Olare Motorogi conservancy
21	LIO51	Female	Cub	Engoyanai	Olare Motorogi conservancy/Naboisho conservancy
22	LIO53	Female	Sub adult	Enesikiria	Naboisho conservancy
23	LIO54	Female	Cub	Engoyanai	Olare Motorogi conservancy/Naboisho conservancy
24	LIO55	Male	Sub adult	Moniko	Olare Motorogi conservancy/Naboisho conservancy
25	LIO57	Female	Cub	Enesikiria	Naboisho conservancy
26	LIO59	Male	Cub	Enesikiria	Naboisho conservancy
27	LIO61	Male	Adult	Double X Males	Maasai Mara National Reserve
28	LIO64	Female	Sub adult	Engoyanai Unicorns	Olare Motorogi conservancy/Naboisho conservancy
29	LIO71	Male	Sub adult	Ol Kinyei DMI	Ol Kinyei conservancy
30	LIO72	Female	Adult	Cheli	Mara North Conservancy
31	LIO73	Male	Sub adult	Cheli	Mara North Conservancy
32	LIO75	Male	Cub	Cheli	Mara North Conservancy
33	LIO76	Female	Adult	Cheli	Mara North Conservancy
34	LIO77	Female	Adult	Cheli	Mara North Conservancy
35	LIO78	Male	Cub	Cheli	Mara North Conservancy
36	LIO81	Female	Adult	Cheli	Mara North Conservancy
37	LIO83	Female	Cub	Cheli	Mara North Conservancy
38	LIO86	Female	Adult	Cheli	Mara North Conservancy

39	LIO87	Male	Sub adult	Cheli	Mara North Conservancy
40	LIO91	Female	Adult	Enesikiria	Naboisho conservancy
41	LIO92	Male	Sub adult	Enesikiria	Naboisho conservancy
42	LIO93	Female	Cub	Enesikiria	Naboisho conservancy
43	LIO94	Male	Sub adult	Enesikiria	Naboisho conservancy
44	LIO95	Male	Cub	Engoyanai	Olare Motorogi conservancy/Naboisho conservancy
45	LIO96	Male	Sub adult	Fig Tree DMI	Maasai Mara National Reserve
46	LIO98	Male	Sub adult	Iikisiusiu	Naboisho conservancy
47	LIO98B	Female	Adult	Iseketa	Olare Motorogi conservancy
48	LIO99	Male	Sub adult	KWS	Maasai Mara National Reserve
49	LIO103	Female	Adult	Marsh	Maasai Mara National Reserve
50	LIO104	Male	Adult	MNC Coalition	Mara North Conservancy
51	LIO105	Female	Adult	Off Beat	Mara North Conservancy
52	LIO106	Female	Adult	Ol Kinyei	Ol Kinyei conservancy
53	LIO107	Female	Adult	Ol Kinyei	Ol Kinyei conservancy
54	LIO109	Male	Sub adult	Porini	Ol Kinyei conservancy
55	LIO110	Male	Sub adult	Purungat	Mara Triangle
56	LIO111	Female	Adult	Sampu Enkare	Naboisho conservancy /Ol Kinyei conservancy
57	LIO112	Male	Sub adult	Unknown	
58	LIO114	Female	Sub adult	Unknown	
59	LIO115	Male	Sub adult	Unknown	
60	LIO116	Female	Sub adult	Unknown	
61	LIO117	Male	Sub adult	Fig-DMI	Maasai Mara National Reserve
62	LIO118	Male	Cub	Engoyanai	Olare Motorogi conservancy/Naboisho conservancy
63	LIO120	Female	Adult	Enesikiria	Naboisho conservancy

Appendix II: The former and revised classification of lion

S/NO	FORMER CLASSIFICATION OF LION	REVISED CLASSIFICATION OF LION
1	Kingdom: Animalia	Kingdom: Animalia
2	Phylum: Chordata	Phylum: Chordata
3	Class: Mammalia	Class: Mammalia
4	Order: Carnivora	Order: Carnivora
5	Family: *Felidae*	Family: *Felidae*
6	Genus: Panthera	Genus: Panthera
7	Species: *Panthera leo*	Species: *Panthera leo*
8	Sub-species:	Sub-species:
	1. *Panthera leo leo*	1. *Panthera leo leo*
	2. Panthera leo persica	*2. Panthera leo melanochaita*

Appendix III: NanoDrop Results of the Samples used in the Study

Sample ID	Conc(ng/ul)	230	A260	280	260/280	260/230	Date/Time
BLANK							31-07-18 3:13
1	40.33	0.947	0.81	0.346	2.33	0.85	31-07-18 3:13
2	23.43	0.676	0.47	0.231	2.03	0.69	31-07-18 3:13
3	61.62	1.289	1.23	0.473	2.6	0.96	31-07-18 3:14
4	2.9	0.281	0.06	0.009	6.14	0.21	31-07-18 3:14
5	65.68	1.671	1.31	0.481	2.73	0.79	31-07-18 3:15
6	167.24	2.411	3.35	1.61	2.08	1.39	31-07-18 3:15
7	45.02	1.298	0.9	0.337	2.67	0.69	31-07-18 3:16
8	160.2	2.309	3.2	1.5	2.14	1.39	31-07-18 3:16
9	52.3	0.925	1.05	0.528	1.98	1.13	31-07-18 3:17
10	84.48	1.518	1.69	0.786	2.15	1.11	31-07-18 3:17
11	20.67	0.979	0.41	0.084	4.91	0.42	31-07-18 3:18
12	58.38	1.326	1.17	0.437	2.67	0.88	31-07-18 3:19
13	12.37	0.329	0.25	0.036	6.82	0.75	31-07-18 3:19
14	88.02	2.533	1.76	0.693	2.54	0.69	31-07-18 3:19
15	33.09	0.997	0.66	0.266	2.49	0.66	31-07-18 3:20
16	3.92	0.213	0.08	0.015	5.07	0.37	31-07-18 3:20
17	17.66	0.656	0.35	0.121	2.93	0.54	31-07-18 3:21
18	61.97	0.942	1.24	0.608	2.04	1.32	31-07-18 3:22
19	54.13	1.314	1.08	0.422	2.57	0.82	31-07-18 3:22
20	156.44	4.406	3.13	0.943	3.32	0.71	31-07-18 3:22
21	149.19	3.683	2.98	1.177	2.54	0.81	31-07-18 3:23
22	131.78	1.877	2.64	1.28	2.06	1.4	31-07-18 3:23
23	7.46	0.748	0.15	0.056	2.69	0.2	31-07-18 3:23
24	135.16	1.749	2.7	1.365	1.98	1.55	31-07-18 3:24
25	54.68	1.242	1.09	0.584	1.87	0.88	31-07-18 3:24
26	39.27	0.894	0.79	0.251	3.13	0.88	31-07-18 3:25
27	26.48	0.917	0.53	0.144	3.67	0.58	31-07-18 3:25
28	41.63	0.815	0.83	0.35	2.38	1.02	31-07-18 3:25
29	1.08	0.088	0.02	0.035	0.62	0.25	31-07-18 3:25
30	37.24	0.529	0.75	0.39	1.91	1.41	31-07-18 3:26
31	3.01	0.128	0.06	0.04	1.5	0.47	31-07-18 3:26
32	8.83	0.467	0.18	0.08	2.21	0.38	31-07-18 3:27
33	156.81	2.505	3.14	1.495	2.1	1.25	31-07-18 3:43
34	33.74	0.548	0.68	0.4	1.69	1.23	31-07-18 3:43
35	74.79	1.413	1.5	0.795	1.88	1.06	31-07-18 3:43
36	157.44	2.432	3.15	1.621	1.94	1.29	31-07-18 3:43
37	30.01	0.861	0.6	0.297	2.02	0.7	31-07-18 3:44
38	37.73	0.364	0.76	0.448	1.68	2.08	31-07-18 3:44
39	69.47	0.771	1.39	0.748	1.86	1.8	31-07-18 3:45
40	33.05	0.428	0.66	0.404	1.64	1.54	31-07-18 3:45
BLANK							**31-07-18 3:45**
41	148.77	1.666	2.98	1.493	1.99	1.79	31-07-18 3:46
42	326.87	7.077	6.54	4.154	1.57	0.92	31-07-18 3:47
43	100.57	1.166	2.01	0.947	2.12	1.73	31-07-18 3:47
44	103.93	1.157	2.08	1.008	2.06	1.8	31-07-18 3:47
45	99.38	2.75	1.99	0.794	2.5	0.72	31-07-18 3:47
46	224.7	4.406	4.49	2.815	1.6	1.02	31-07-18 3:48
47	383.53	5.63	7.67	4.429	1.73	1.36	31-07-18 3:48
48	127.22	1.58	2.54	1.282	1.98	1.61	31-07-18 3:48
49	40.55	0.483	0.81	0.416	1.95	1.68	31-07-18 3:49
50	64.11	1.002	1.28	0.648	1.98	1.28	31-07-18 3:50
51	50.85	0.762	1.02	0.469	2.17	1.33	31-07-18 3:50

Sample ID	Conc(ng/ul)	230	A260	280	260/280	260/230	Date/Time
52	130.8	1.953	2.62	1.178	2.22	1.34	31-07-18 3:50
53	84.92	0.973	1.7	0.901	1.88	1.75	31-07-18 3:51
54	61.24	0.632	1.23	0.629	1.95	1.94	31-07-18 3:51
55	113.35	1.474	2.27	1.175	1.93	1.54	31-07-18 3:51
56	817.97	17.03	16.4	9.99	1.64	0.96	31-07-18 3:51
57	80.26	0.98	1.61	0.799	2.01	1.64	31-07-18 3:52
58	106.97	1.924	2.14	0.973	2.2	1.11	31-07-18 3:52
59	73.74	0.653	1.48	0.676	2.18	2.26	31-07-18 3:53
60	46.04	0.614	0.92	0.467	1.97	1.5	31-07-18 3:53
61	45.5	0.859	0.91	0.443	2.05	1.06	31-07-18 3:53
62	83.44	1.746	1.67	0.681	2.45	0.96	31-07-18 3:54
63	84.06	1.508	1.68	0.779	2.16	1.11	31-07-18 3:54
64	98.11	1.668	1.96	0.947	2.07	1.18	31-07-18 3:54
65	67.81	0.987	1.36	0.69	1.97	1.37	31-07-18 3:55
66	96.56	1.703	1.93	0.775	2.49	1.13	31-07-18 3:55
67	83.01	1.735	1.66	0.661	2.51	0.96	31-07-18 3:55
68	81.2	1.983	1.62	0.427	3.81	0.82	31-07-18 3:56
69	36.5	0.654	0.73	0.382	1.91	1.12	31-07-18 3:56
70	142.56	4.234	2.85	0.521	5.47	0.67	31-07-18 3:56
71	55.29	0.563	1.11	0.565	1.96	1.96	31-07-18 3:56
72	42.64	0.604	0.85	0.409	2.08	1.41	31-07-18 3:57
73	2.69	0.312	0.05	0.03	1.79	0.17	31-07-18 3:57
74	10.23	0.459	0.21	0.099	2.07	0.45	31-07-18 3:58
75	40.27	0.891	0.81	0.291	2.76	0.9	31-07-18 3:58
76	21.82	0.541	0.44	0.208	2.1	0.81	31-07-18 3:58
77	51.48	0.796	1.03	0.534	1.93	1.29	31-07-18 3:58
78	93.33	1.285	1.87	0.97	1.92	1.45	31-07-18 3:59
79	176.2	2.86	3.52	1.487	2.37	1.23	31-07-18 3:59
80	180.44	2.053	3.61	1.844	1.96	1.76	31-07-18 3:59
BLANK							31-07-18 4:00
81	73.24	-0.081	1.47	0.768	1.91	-18.19	31-07-18 4:01
82	563.86	5.492	11.3	6.175	1.83	2.05	31-07-18 4:01
83	195.42	0.869	3.91	1.971	1.98	4.5	31-07-18 4:01
84	152.68	0.37	3.05	1.532	1.99	8.24	31-07-18 4:02
85	150.94	0.411	3.02	1.525	1.98	7.34	31-07-18 4:02
86	141.66	0.725	2.83	1.291	2.19	3.91	31-07-18 4:02
87	149.49	0.227	2.99	1.539	1.94	13.18	31-07-18 4:02
88	88.21	-0.421	1.76	0.959	1.84	-4.19	31-07-18 4:03
89	141.95	1.433	2.84	1.288	2.2	1.98	31-07-18 4:03
90	269.07	3.587	5.38	3.314	1.62	1.5	31-07-18 4:04
91	457.37	7.872	9.15	5.725	1.6	1.16	31-07-18 4:04
92	48.92	-0.402	0.98	0.452	2.17	-2.44	31-07-18 4:05
93	53.53	-1.152	1.07	-2.817	-0.38	-0.93	31-07-18 4:05
93	50.73	0.053	1.02	0.423	2.4	19.02	31-07-18 4:06
94	31.34	-0.947	0.63	0.342	1.83	-0.66	31-07-18 4:06
95	1.29	-1.127	0.03	0.027	0.96	-0.02	31-07-18 4:06
95	0.37	-1.121	0.01	0.032	0.23	-0.01	31-07-18 4:07
96	6.45	-0.966	0.13	0.065	1.99	-0.13	31-07-18 4:07
97	129.35	1.123	2.59	1.05	2.46	2.3	31-07-18 4:07
98	403.94	3.4	8.08	4.437	1.82	2.38	31-07-18 4:08
98B	44.29	-0.662	0.89	0.485	1.83	-1.34	31-07-18 4:08
99	42.68	-0.681	0.85	0.48	1.78	-1.25	31-07-18 4:08
100	0.95	-0.843	0.02	0.04	0.48	-0.02	31-07-18 4:09
101	250.13	1.622	5	2.592	1.93	3.08	31-07-18 4:09
101	239.41	1.399	4.79	2.433	1.97	3.42	31-07-18 4:09
102	519.57	3.588	10.4	5.425	1.92	2.9	31-07-18 4:10
103	2.64	-1.174	0.05	0.038	1.39	-0.05	31-07-18 4:10
104	16.01	-0.929	0.32	0.204	1.57	-0.34	31-07-18 4:11

Sample ID	Conc(ng/ul)	230	A260	280	260/280	260/230	Date/Time
105	82.48	-0.406	1.65	0.885	1.86	-4.07	31-07-18 4:11
106	286.19	2.82	5.72	4.256	1.35	2.03	31-07-18 4:11
107	6.19	-1.034	0.12	0.068	1.82	-0.12	31-07-18 4:12
108	104.26	0.976	2.09	0.774	2.7	2.14	31-07-18 4:13
109	67.56	-0.516	1.35	0.717	1.88	-2.62	31-07-18 4:13
110	24.08	-0.981	0.48	0.26	1.85	-0.49	31-07-18 4:13
111	3.68	-0.907	0.07	0.053	1.39	-0.08	31-07-18 4:14
BLANK							31-07-18 4:14
112	16.38	0.353	0.33	0.166	1.97	0.93	31-07-18 4:14
113	82.23	1.04	1.65	0.757	2.17	1.58	31-07-18 4:15
114	21.86	0.482	0.44	0.187	2.34	0.91	31-07-18 4:15
115	99.57	1.611	1.99	0.932	2.14	1.24	31-07-18 4:15
116	200.46	1.97	4.01	1.954	2.05	2.04	31-07-18 4:16
117	45.22	0.581	0.9	0.404	2.24	1.56	31-07-18 4:16
118	33.85	0.31	0.68	0.321	2.11	2.19	31-07-18 4:16
120	23.52	2.441	0.47	0.308	1.53	0.19	31-07-18 4:17

Appendix IV: Fasta format of *Panthera Leo* isolates 89_Kenya mitochondrion, complete genome: GenBank: KP001498.1 (Source: NCBI Gene Bank)

>KP001498.1 Panthera leo isolate 89_Kenya mitochondrion, complete genome

GGGTTAATGACTAATCAGCCCATGATCACACATAACTGTGGTGTCATGCATTTGGTATCTTTAATTTTTTGGGGG
GTCGAACTTGCTATGACTCAGCTATGACCTAAAGGTCCTGACTCAGTCAAATATAATGTAGCTGGGCTTATTCTC
TATGCGGGGGTTCCACACGTACAACAAACAAGGTGTTATTCAGTCAATGGTCACAGGACATATACTTAAATCCC
TATTGCTCCACAGGACACGGCGAGCGCGCACCCACGTATACGCGCACATGTGTACACACGTACACACGTACAC
GTACACACGTACACGTACACACGTACACGTACACACGTACACGTACACACGTACACGTACACACGTACACACG
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CACGTATACACGTACACACGTACACGTACACGTACACGTACACGTACACGTACACGTACACGTACACGTACAC
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GTGCCAGCCACCGCGGTCATACGATTAACCCAGACTAATAGACTTACGGCGTAAAGCGTGTTACAGAAGAAAA

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TGCTTTACATCCCTCTAGAGGAGCCTGTTCTATAATCGATAAACCCCGATAAACCTCACCATCTCTTGCTAATTC

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GTTAGGTCAAGGTGTAGCCTATGAGATGGGAAGCAATGGGCTACATTTTCTACAATTAGAACACCCACGAAAA

TCCTTATGAAACTAAGCATTCAAGGAGGATTTAGCAGTAAATTTGAGAATAGAGAGCTCAATTGAATCGGGCC

ATGAAGCACGCACACACCGCCCGTCACCCTCCTCAAGTGACTAGCCCCTAAAGAAACCTATTCAAACCACTACA

TCCACAAGAGGAGACAAGTCGTAACAAGGTAAGCATACTGGAAAGTGTGCTTGGATGACAAGATGTAGCTTAA

ACTAAAGCGTCTGGCTTACACCCAGAAGATTTCATATTAAACTGACCGTCTTGAGCCAAAGCTAGCCCAATCAT

CTACAAACGCAACTAACACTAGAAAGTAAAATAAAACATTTAGTTACCCCATAAAAGTATAGGAGATAGAAAT

TTAACTTGGCGCTATAGAGAAAGTACCGCAAGGGAAGGATGAAAGAAAAAACTAAAAGCACTATACAGCAAA

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TTACCAAAGCGCCTTTAACCAAATAGATGATATAATCTCAATCTAGACAGTTTATCTAAACACATCGCCCGAGA

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CGCCCTATTCTTTTTAGCAGAATATGCCAACATTATCATAATAAACATCCTCACAACAATCCTGTTCTTCGGAGC

ATTTCATAGTCCCTACATACCAGAACTATATACCACTAACTTCACCGTAAAAACCCTGATCTTAACAACCACCTT

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CCCTTACCCTAGCTCTATGTATATGGCACGTCTCCCTACCCATTATCACAGCAAGTATCCCACCTCAAACATAAG

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CACTCAGAACAATGAACCGTATCAAAGGATCTTAACCCCATAGCATCCATCGTAATAACAACCGCCCTAGCAAT

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TAAACCTAACCATCTACATCACCATAACACTAACCACCTTCATACTATTCATATACAACTCCACCACAACAACA

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GGCCTTCCTCCGCTCTCCGGCTTTATCCCAAAATGAATAATCATTCAAGAACTAACTAAAAATGAAATAATTAT

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TTCTCAAATAGGACATCTCGA

Appendix V: Fasta format of *Panthera Leo* isolates 89_Kenya Control Region

GenBank: KP001498.1 (Source: NCBI Gene Bank)

>KP001498.1:16396-17119, 1-944 Panthera leo isolate 89_Kenya Control Region

GTCATGCATTTGGTATCTTTAATTTTTTGGGGGGTCGAACTTGCTATGACTCAGCTATGACCTAAAGGTCCTGAC
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GAACGATATTTACACGCCCAACAATCCATCTTG

Appendix VI: Procedure for measuring DNA quality and purity

The measurement of the DNA quantity and quality included the following procedure involving the below seven steps, logging on to the computer, selecting the program, cleaning the lenses, initializing the system, Blanking, Running the sample, Saving, exporting and Retrieving the Data. .After logging on to the computer system, the program Nano Drop 2000 was selected, double clicked and Nucleic acids icon was selected since the research was working on the DNA. The top and lower lenses were cleansed and 1-2 µl of distilled water was aliquoted on to the top lenses and the Pedestal (Lower area) so that the water would form a column between the upper and the lower lenses. The water was then blotted off using the Kimwipe absorbent towels. Then the machine was initialized by putting an aliquot of 1 µl of distilled water on the lower lens followed by closure of the machine arm and initialization button clicked on. Once initialization was complete, the arm was raised and lens blotted with a Kimwipe.

The blanking process was done using eluting buffer by loading 1 µl of eluting buffer used to dissolve the DNA on to the bottom lens, the arm was closed and the blank menu clicked. The arm was then raised again and the lens blot using the Kimwipe. The sample was vortexed by finger flicking the base of the sample tube 4-5 times to achieve homogeneity. Then 1 µl of DNA sample was aliquoted into the Pedestal unit and the arm closed. The sample ID was typed and repeated for every DNA sample and the measure button pressed to start the analysis. The program created the spreadsheet that was exported to the excel sheet and used to record the various measurements including the sample ID, 260/280, 260/230 ratio and the concentration in ng/µl. Re-blanking was repeated after every 39 samples using the eluting Buffer. Finally, the top and bottom lenses were then cleansed using distilled water and blot-dried using the Kimwipe absorbent towels. The data was retrieved by clicking the Menu,

Show Reports, then click on Reports on the top menu, save Report, export Report table only, name the file and save menu. Then the Nano Drop program was exited and the results attached in the email and sent to my file.

Appendix VII: Procedure for preparing PCR Master Mix

All materials that were required for preparing master mix for PCR including nuclease free double distilled water, 10x PCR buffer, 10mM dNTPs, 50mM Magnesium Chloride ions, both Forward and Reverse primers and Taq Polymerase were assembled and the sterile 2.0 ml eppendorff tubes were well labled ready for use. All these materials were removed from -80°C and placed in a tray of ice pack to maintain their condition at very low temperatures.

Appendix VIII: Procedure for preparing working dNTPs

Using a sterile 1.5 ml eppendorff tube, a 100 µl of each 100 mM dNTP (dATP, dTTP, dCTP, and dGTP) (Catalogue number BIO-39049) was added in to the tube using a P200 micropipette and sterile filter tips. Using a P1000 micropipette and sterile tips, 400 µl of sterile pure nuclease free double distilled water was added to make a final concentration of 10 millimolar (10 mM) dNTPs. The mixture was slightly vortexed to mix and then stored at -80°C until use.

Appendix IX: Procedure for Primer Reconstitution

Both forward and reverse high fidelity platinum primers were supplied in 40.6 nanomoles of 20 bp and 39.0 nanomoles of 20 bp respectively. To make a 100 micromolar stock solution, A total volume of 406 µl of nuclease free water was added to a 40.6 nanomoles of 21 bp forward

primer and 390 µl nuclease free water was added to a 39.0 nanomoles of 19 bp reverse primer and the mixture slightly vortexed to make the stock solution. This was equivalent to a 100 micro-molar (µM) stock solution. To prepare 10 micro-molar of the working solution primers, 10 µl of the stock was pipetted using a P 20 micropipette and mixed with 90 µl of nuclease free double distilled water pipetted using a p100/200 micropipette. Both the stock and working solution primers were kept at -80°C deep freezer until use.

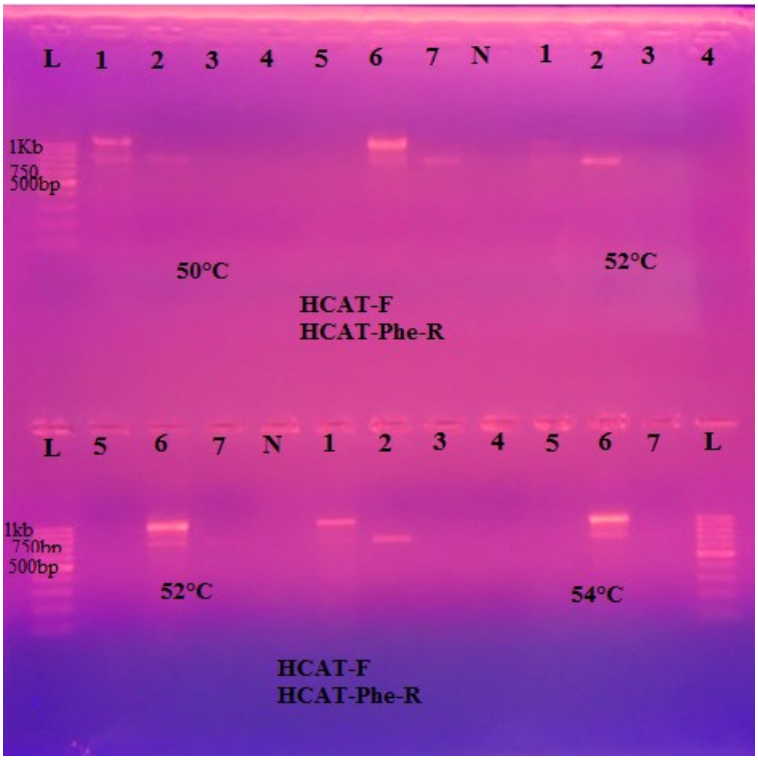
To make a 100 micromolar stock solution, 40.6 nanomoles of forward primer and 39.0 nanomoles of reverse primer was converted into micromoles as below:

Forward primer	Reverse primer
40.6 nmoles x 1 µmole/1000 nmole = 0.0406 µmole,	39.0 nmoles x 1 µmole/1000 nmole = 0.39 µmole,
0.0406 µmole/100 µmole/litre = 0.00046 L	0.039 µmole/100 µmole/litre = 0.00039 L
0.000406 L x 1000 m/L = 0.406 ml or 406 µl	0.00039 L x 1000 m/L = 0.39 ml or 390 µl

Appendix X: Procedure for Primer optimization

The forward and reverse primers for the lion mitochondrial D-loop region were both synthesized as per Dubach et al. 2005 at Inqaba Biotech Technologies in South Africa. In order to verify the specificity of the reaction by optimization, gradient PCR reaction was carried out in a SimpliAmp Thermal Cycler from Applied Biosystems by Thermo Fisher Scientific (SN 228007943) using seven samples at different annealing temperatures of 50 °C, 52 °C and 54 °C for 45 s with other conditions held at constant temperatures of 94 °C for 1 min (Initial denaturation), 94 °C for 40 s (Second denaturation), 72 °C for 45 s (Initial

extension) (35 cycles) and a final extension at 72 °C for 10 min. The PCR product was obtained at the annealing temperature of 52 °C. All the used annealing temperatures gave almost similar results, however the annealing temperature of 52 °C was selected to be used for subsequent PCR reactions in this study because it has been confirmed by Dubach et al,. 2005 to be the optimum annealing temperature. To optimize the primers used in this study, seven samples were amplified in a gradient PCR at different Annealing temperatures of 50°C, 52°C, and 54°C. The temperature that gave the best bands was taken as the appropriate annealing temperature to use in this study. In this study 52°C was used.



PCR bands using gradient PCR for primer optimization

Appendix XI: Procedure for preparing TE Buffer

Using a clean 50 ml measuring cylinder, 10 ml of 1 M Tris-Cl was measured and transferred to a clean beaker labeled TE buffer. 2.0 ml of 0.5 M EDTA was then added. Using a 1000 ml

measuring cylinder, 600 ml of distilled water was added and mixed using a clean glass rod. The solution was transferred to a 1000 Ml measuring cylinder and then adjusted to 1000 Ml. the final solution was stored in a clean reagent bottle labled TE Buffer at room temperature until use.

Appendix XII: Procedure for preparing 10x TAE Buffer

To prepare 10x TAE buffer, 48.4 g of Tris base was added to a 1000 ml empty beaker labeled 10x TAE buffer. 11.4 ml glacial acetic acid in a 50 ml measuring cylinder was added. 20 ml of 0.5 M EDTA was then added to the mixture using the measuring cylinder. Using a 1000 ml beaker, 600 ml of distilled water was then added and the solution gently mixed with a clean glass rod. The volume of the buffer was adjusted to 1000 ml and stored in a clean reagent bottle labled 10x TAE Buffer at room temperature until use.

Appendix XIII: Procedure for preparing 5x TBE Buffer

Using a clean spatula, 54.0 g of Tris-base was weighed in a well calibrated analytical balance and emptied in a clean 1000 ml beaker labeled 5x TBE buffer. Using a clean spatula, 27.5 g of boric acid was weighed and added to the mixture, stirred and then well mixed. Using a clean 1000 ml measuring cylinder, 600 ml of distilled water was added, well mixed with a clean glass rod and the solution transferred to a clean 1000 ml measuring cylinder. The volume was adjusted to 1000 ml and the buffer stored in a clean reagent bottle labeled 5x TBE buffer.

Appendix XIV: Procedure for making DNA loading dye

A total of 0.25 gm of Bromophenol blue was dissolved in 30 ml of glycerol in 50 ml of

distilled water. The pH was adjusted to 8.0 by addition of 10N NaOH. The volume was then adjusted to 100 ml with double distilled water and the final solution was kept at 4°C until use.

Appendix XV: Procedure for Gel preparation

All materials required for gel preparation including double distilled water, Tris Base, 0.5M EDTA, glacial Acetic acid, Pure Grade Analytical Agarose powder and Ethidium Bromide were assembled in order to prepare the gel for electrophoresis. All other machines and apparatus were also assembled and ensured that they were in good working condition.

A 1.5% gel concentration was used in this study because the length of the mitochondrial D-loop region to be isolated was approximately 1000 bps long. Exactly 2.1 g of pure analytical grade Top Vision Agarose powder (Catalogue number R0492) was measured on a well calibrated Sartorius analytical balance (Thermo Fisher Scientific, USA) using a clean plastic weighing boat and put in a clean conical flask. Using a 200 ml measuring cylinder, 140 ml of Tris Borate EDTA (TBE) buffer was measured and added into the beaker. The mixture was then boiled in a Manumaster commercial microwave (Thermo Fisher Scientific, USA) oven 2100 for 1 min, 45 s to a 100°C and then slightly cooled to approximately 60°C-70°C while carefully swirling in a running tap water. Care was taken to ensure no bubbles formed so as not to interfere with the movement of the DNA during electrophoresis. After cooling the gel for a while, 6.0 µl of Ethidium Bromide was mixed with 140 ml gel and swirled for a while to achieve homogeneity and maximal staining with Ethidium Bromide. The liquid gel was then carefully and gently poured into the prior assembled 140 ml volume gel tank with combs in place. The gel was left for approximately 30-60 min to polymerize. After polymerization, the combs were carefully and gently removed from the gel leaving sample wells. Carefully, the

gel was transferred to an electrophoresis tank full of TBE electrophoresis buffer that completely covered the wells.

Appendix XVI: Procedure for Sample loading and Gel electrophoresis

Using a well calibrated P20 Finn micropipette, 25 µl of amplified PCR product and 3 µl of 6x DNA loading dye (Blue) were pipetted and well mixed in a clean sterile para-film paper. Using a well calibrated P10 Finn micropipette and clean sterile micropipette tips, 3 µl of 100 bp DNA ladder was first loaded onto the first and the last wells of gel in the electrophoresis tank. The PCR products were then loaded on the subsequent wells and the negative control (PCR master mix) was then loaded into the second last well. The electrophoresis tank was then connected to the power supply at 100 Voltage for 2 hours. The process was closely monitored to ensure that neither the DNA ladder nor the PCR products overran.

Appendix XVII: Procedure for Gel Purification

After the gel was viewed on the gel documentation system, the 0.1 Kb bands were identified and the band of interest cut using a sterile surgical blade number 21. The bands were excised as close to the fragment as possible to minimize the size of the gel. The bands were then put into a pre-weighed clean sterile 1.5 ml eppendorff tube weighing 1gm. The tube and the cut fragment were weighed in a well calibrated Sartorius analytical weighing balance. To obtain the weight of the fragment, the weight of the empty tube (1gm) was deducted from the total weight. The weight was noted and the weighed gel with the fragment was then used in the subsequent gel purification procedure. The weight of the gel was used to measure the volume of the binding buffer during gel purification. The excised gel with the DNA fragment was

purified using the GeneJET Gel Extraction Kit (#K0691) from Thermo Fisher Scientific (http://www.thermoscientifc.com/onebio) using the Manufacturer's instructions. The Thermo Scientific GeneJET Gel Extraction kit (#K0692) contained the following contents: 150 ml Binding Buffer (Yellow), 45 ml of concentrated Wash Buffer to be diluted with Absolute or 96% Ethanol (Colorless), 30 ml Elution Buffer which contains 10mM Tris Hcl at pH 8.5 (colorless) and 250 GeneJET Purification columns pre-assembled with 1.5 ml collection tubes. 1:1 volume of Binding Buffer was added to the gel slice (Volume: Weight). In this study, most gel slice weighed 300 gm; therefore where the gel was 300 gm, 300 µl of Binding Buffer was added to 300 gm of gel slice. The gel mixture was then incubated using floaters at 60°C for 10 min or until the gel was completely dissolved in a water bath. To facilitate easy melting of the gel slice, the contents of the tubes were periodically inverted to mix every few minutes. The mixture was then briefly vortexed and checked for yellow color which is an indicator of maximum DNA binding. A volume of 10 µl of 3M sodium acetate at pH 5.2 was added and mixed in case the color of the solution was orange or violet for the color of the mixture to turn to yellow.

The total content of the solubilized gel solution in the tube was then transferred to the GeneJET purification column. The contents were then centrifuged in an eppendorff microcentrifuge 5425 (SN: 5405HL601920) type 5425 (Germany) for 1 min. The flow through was discarded and then the same collection tube placed back to the column. Since the DNA was to be used for sequencing, 100 µl of binding buffer was added into the GeneJET purification column. The columns' were then spinned for 1 minute and the flow through poured again. The columns were then placed back to the same collection tubes. Total volume of diluted 700 µl Washing Buffer was added to the GeneJET columns and spinned for another

1 minute. The flow through was then poured and columns placed back to the same collection tubes. The GeneJET purification columns with intact DNA were then centrifuged for an extra 1 minute to remove the residue washing buffer. The GeneJET purification columns with intact DNA were then transferred to 1.5 µl microcentrifuge tubes and 30 µl of Elution Buffer added to elute the DNA. The GeneJET purification column was centrifuged for 1 min and the GeneJET purification column discarded. The eluted DNA in the 1.5 microcentrifuge tube was then stored at -20 °C for subsequent sequencing procedures.

Appendix XVIII: % Identity matrix showing identities of 56 lion DNA sequences

	LIO120_...	LIO118b...	LIO117_...	LIO116_...	LIO115_...	LIO114_...	LIO112_...	LIO111_...	LIO110_...	LIO109_...	LIO107_...	LIO106_...	LIO105_...	LIO104_...	LIO103_...	LIO99_K...	LIO98B_I...	LIO98_IT...	LIO95_E...	LIO94_E...	LIO93_E...	LIO92_E...	LIO91_E...
LIO120_Eneskiria_Female_...		99.65%	98.84%	99.65%	99.42%	99.30%	99.53%	99.65%	99.42%	99.07%	99.53%	98.14%	98.03%	98.03%	98.03%	99.19%	99.18%	99.65%	98.14%	98.37%	90.25%	99.42%	99.77%
LIO118b_Engoyanai_Male_Cub	99.65%		99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO117_Flg_DMI_Male_Sub...	98.84%	99.19%		99.19%	98.95%	98.84%	99.07%	98.49%	98.95%	98.37%	99.07%	98.60%	97.80%	97.80%	97.80%	98.95%	98.72%	99.19%	99.07%	97.91%	89.71%	99.19%	98.84%
LIO116_Unknown_Male_Su...	99.65%	100%	99.19%		99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO115_Unknown_Male_Su...	99.42%	99.77%	98.95%	99.77%		99.65%	99.65%	99.07%	99.53%	98.95%	99.65%	98.26%	98.15%	98.15%	98.15%	99.30%	99.30%	99.77%	98.26%	98.49%	90.14%	99.53%	99.42%
LIO114_Unknown_Female_...	99.30%	99.65%	98.84%	99.65%	99.65%		99.53%	98.95%	99.42%	98.83%	99.53%	98.14%	98.04%	98.04%	98.04%	99.19%	99.18%	99.65%	98.14%	98.38%	90.26%	99.42%	99.30%
LIO112_Unknown_Male_Su...	99.53%	99.88%	99.07%	99.88%	99.65%	99.53%		99.18%	99.65%	99.07%	99.77%	98.37%	98.27%	98.27%	98.27%	99.42%	99.42%	99.88%	98.37%	98.84%	90.25%	99.65%	99.53%
LIO111_Sampu_Enkare_Fe...	99.65%	99.30%	98.49%	99.30%	99.07%	98.95%	99.18%		99.07%	98.72%	99.18%	97.79%	97.69%	97.69%	97.69%	98.84%	98.83%	99.30%	97.79%	98.03%	90.15%	99.07%	99.42%
LIO110_Purungat_Male_Su...	99.42%	99.77%	98.95%	99.77%	99.53%	99.42%	99.65%	99.07%		98.95%	99.65%	98.26%	98.15%	98.15%	98.15%	99.30%	99.77%	99.77%	98.26%	98.95%	90.14%	99.53%	99.65%
LIO109_Porini_Male_Sub_adult	99.07%	99.18%	98.37%	99.18%	98.95%	98.83%	99.07%	98.72%	98.95%		99.07%	98.49%	97.69%	97.69%	97.69%	98.72%	98.72%	99.18%	98.49%	97.91%	89.57%	98.95%	99.07%
LIO107_Ol_Kinyei_Female_...	99.53%	99.88%	99.07%	99.88%	99.65%	99.53%	99.77%	99.18%	99.65%	99.07%		98.60%	98.27%	98.27%	98.27%	99.42%	99.42%	99.88%	98.60%	98.61%	90.02%	99.65%	99.53%
LIO106_Ol_Kinyei_Female_...	98.14%	98.49%	98.60%	98.49%	98.26%	98.14%	98.37%	97.79%	98.26%	98.49%	98.60%		97.58%	97.58%	97.58%	98.49%	98.02%	98.49%	99.53%	97.22%	88.93%	98.26%	98.14%
LIO105_Off_beat_Female_...	98.03%	98.38%	97.80%	98.38%	98.15%	98.04%	98.27%	97.69%	98.15%	97.69%	98.27%	97.58%		100%	100%	98.84%	97.92%	98.38%	97.34%	97.12%	88.99%	98.15%	98.03%
LIO104_MNC_Coalition_Mal...	98.03%	98.38%	97.80%	98.38%	98.15%	98.04%	98.27%	97.69%	98.15%	97.69%	98.27%	97.58%	100%		100%	98.84%	97.92%	98.38%	97.34%	97.12%	88.99%	98.15%	98.03%
LIO103_Marsh_Female_Adult	98.03%	98.38%	97.80%	98.38%	98.15%	98.04%	98.27%	97.69%	98.15%	97.69%	98.27%	97.58%	100%	100%		98.84%	97.92%	98.38%	97.34%	97.12%	88.99%	98.15%	98.03%
LIO99_KWS_Male_Sub_adult	99.19%	99.53%	98.95%	99.53%	99.30%	99.19%	99.42%	98.84%	99.30%	98.72%	99.42%	98.49%	98.84%	98.84%	98.84%		99.07%	99.53%	98.26%	98.26%	89.93%	99.30%	99.19%
LIO98B_Iseketa_Female_Adult	99.18%	99.53%	98.72%	99.53%	99.30%	99.18%	99.42%	98.83%	99.77%	98.72%	99.42%	98.02%	97.92%	97.92%	97.92%	99.07%		99.53%	98.02%	98.72%	89.91%	99.30%	99.42%
LIO98_Ilkisiusiu_Male_Adult	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%		98.49%	98.72%	90.14%	99.77%	99.65%
LIO95_Engoyanai_Male_Cub	98.14%	98.49%	99.07%	98.49%	98.26%	98.14%	98.37%	97.79%	98.26%	98.49%	98.60%	99.53%	97.34%	97.34%	97.34%	98.26%	98.02%	98.49%		97.22%	89.04%	98.26%	98.14%
LIO94_Eneskiria_Male_Sub...	98.37%	98.72%	97.91%	98.72%	98.49%	98.38%	98.84%	98.03%	98.95%	97.91%	98.61%	97.22%	97.12%	97.12%	97.12%	98.26%	98.72%	98.72%	97.22%		89.39%	98.49%	98.61%
LIO93_Eneskiria_Female_Cub	90.25%	90.14%	89.71%	90.14%	90.14%	90.26%	90.25%	90.15%	90.14%	89.57%	90.02%	88.93%	88.99%	88.99%	88.99%	89.93%	89.91%	90.14%	89.04%	89.39%		89.91%	90.48%
LIO92_Eneskiria_Male_Sub...	99.42%	99.77%	99.19%	99.77%	99.53%	99.42%	99.65%	99.07%	99.53%	98.95%	99.65%	98.26%	98.15%	98.15%	98.15%	99.30%	99.30%	99.77%	98.26%	98.49%	89.91%		99.42%
LIO91_Eneskiria_Female_A...	99.77%	99.65%	98.84%	99.65%	99.42%	99.30%	99.53%	99.42%	99.65%	99.07%	99.53%	98.14%	98.03%	98.03%	98.03%	99.19%	99.42%	99.65%	98.14%	98.61%	90.48%	99.42%	
LIO87_Cheli_Male_Sub_adult	99.42%	99.77%	98.95%	99.77%	99.53%	99.42%	99.65%	99.07%	99.53%	98.95%	99.65%	98.49%	98.38%	98.38%	98.38%	99.53%	99.30%	99.77%	98.26%	98.49%	90.14%	99.53%	99.42%
LIO86_Cheli_Female_Adult	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO78_Cheli_Female_Adult	98.95%	99.30%	98.49%	99.30%	99.07%	98.95%	99.42%	98.60%	99.53%	98.48%	99.18%	97.79%	97.69%	97.69%	97.69%	98.84%	99.53%	99.30%	97.79%	98.95%	90.02%	99.07%	99.18%
LIO75_Cheli_Male_Cub	98.14%	98.49%	97.90%	98.49%	98.72%	98.60%	98.60%	97.79%	98.72%	97.67%	98.37%	97.44%	97.57%	97.57%	97.57%	98.72%	98.72%	98.49%	97.21%	98.15%	89.71%	98.25%	98.37%
LIO73_Cheli_Male_Sub_adult	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO72_Cheli_Female_Adult	98.48%	98.83%	98.02%	98.83%	99.07%	98.95%	98.95%	98.14%	99.07%	98.02%	98.72%	97.33%	97.23%	97.23%	97.23%	98.37%	99.07%	98.83%	97.33%	98.49%	90.02%	98.60%	98.72%
LIO71_Ol_Kinyei_Male_Sub...	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO64_Engoyanai_Unicorns...	98.14%	98.49%	98.60%	98.49%	98.26%	98.14%	98.37%	97.79%	98.26%	98.49%	98.60%	100%	97.58%	97.58%	97.58%	98.49%	98.02%	98.49%	99.53%	97.22%	88.93%	98.26%	98.14%
LIO61_Double_X_males_M...	99.42%	99.77%	99.19%	99.77%	99.53%	99.42%	99.65%	99.07%	99.53%	98.95%	99.88%	98.49%	98.15%	98.15%	98.15%	99.30%	99.30%	99.77%	98.49%	98.49%	89.91%	99.77%	99.42%
LIO59_Eneskiria_Male_Cub	99.77%	99.65%	98.84%	99.65%	99.42%	99.30%	99.53%	99.42%	99.42%	99.07%	99.53%	98.14%	98.27%	98.27%	98.27%	99.42%	99.18%	99.65%	98.14%	98.37%	90.48%	99.42%	99.77%
LIO57_Eneskiria_Female_Cub	99.30%	99.65%	98.84%	99.65%	99.42%	99.30%	99.53%	98.95%	99.42%	98.83%	99.53%	98.14%	98.03%	98.03%	98.03%	99.19%	99.18%	99.65%	98.14%	98.37%	89.80%	99.42%	99.30%
LIO55_Moniko_Male_Sub_adult	99.07%	99.42%	98.84%	99.42%	99.18%	99.07%	99.30%	98.72%	99.18%	98.60%	99.53%	98.37%	97.80%	97.80%	97.80%	98.95%	98.95%	99.42%	98.14%	98.14%	89.57%	99.42%	99.07%
LIO54_Engoyanai_Female_Cub	99.30%	99.65%	99.07%	99.65%	99.42%	99.30%	99.53%	98.95%	99.42%	98.95%	99.77%	98.84%	98.27%	98.27%	98.27%	99.19%	99.18%	99.65%	98.84%	98.37%	89.80%	99.42%	99.30%
LIO53_Eneskiria_Female_S...	99.18%	99.53%	98.72%	99.53%	99.30%	99.18%	99.65%	98.83%	99.77%	98.72%	99.42%	98.02%	97.92%	97.92%	97.92%	99.07%	99.53%	99.53%	98.02%	99.19%	90.14%	99.30%	99.42%
LIO51_Engoyanai_Female_Cub	98.14%	98.49%	98.60%	98.49%	98.26%	98.14%	98.37%	97.79%	98.26%	98.49%	98.60%	100%	97.58%	97.58%	97.58%	98.49%	98.02%	98.49%	99.53%	97.22%	88.93%	98.26%	98.14%
LIO49_Moniko_Female_Sub...	99.07%	99.42%	98.61%	99.42%	99.19%	99.07%	99.30%	98.72%	99.19%	98.61%	99.30%	97.92%	97.81%	97.81%	97.81%	98.96%	98.95%	99.42%	97.92%	99.07%	89.62%	99.19%	99.07%
LIO48_Engoyanai_Unicorns...	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO45_Moniko_Male_Sub_adult	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO43_Moniko_Female_Adult	99.65%	100%	99.19%	100%	99.77%	99.65%	99.88%	99.30%	99.77%	99.18%	99.88%	98.49%	98.38%	98.38%	98.38%	99.53%	99.53%	100%	98.49%	98.72%	90.14%	99.77%	99.65%
LIO41_Engoyanai_Male_Adult	98.14%	98.49%	98.60%	98.49%	98.26%	98.14%	98.37%	97.79%	98.26%	98.49%	98.60%	100%	97.58%	97.58%	97.58%	98.49%	98.02%	98.49%	99.53%	97.22%	88.93%	98.26%	98.14%
LIO39_Engoyanai_Female_...	99.53%	99.88%	99.07%	99.88%	99.65%	99.53%	99.77%	99.19%	99.65%	99.07%	99.77%	98.37%	98.27%	98.27%	98.27%	99.42%	99.42%	99.88%	98.37%	98.61%	90.03%	99.65%	99.53%
LIO38_Double_X_males_M...	99.53%	99.65%	98.84%	99.65%	99.42%	99.30%	99.53%	99.19%	99.42%	99.07%	99.53%	98.14%	98.04%	98.04%	98.04%	99.19%	99.19%	99.65%	98.14%	98.38%	90.05%	99.42%	99.53%
LIO32_Shampole_Female_5Yrs	98.95%	99.30%	99.42%	99.30%	99.07%	98.95%	99.19%	98.61%	99.07%	98.49%	99.19%	98.84%	97.92%	97.92%	97.92%	99.07%	98.84%	99.30%	98.84%	98.03%	89.72%	99.07%	98.95%
LIO30_Maralal_Female_2Yrs	95.26%	95.49%	94.91%	95.49%	95.26%	95.19%	95.38%	94.92%	95.26%	94.91%	95.38%	94.23%	94.37%	94.37%	94.37%	95.49%	95.03%	95.49%	94.23%	94.91%	87.08%	95.26%	95.14%
LIO29_Waso_Male	94.91%	95.14%	94.57%	95.14%	94.91%	94.80%	95.03%	94.57%	94.91%	94.57%	95.03%	93.88%	94.03%	94.03%	94.03%	95.14%	94.88%	95.14%	93.88%	94.57%	86.63%	94.91%	94.80%
LIO16_Shampole_Male_4yrs	98.48%	98.83%	98.25%	98.83%	98.60%	98.48%	98.72%	98.14%	98.60%	98.02%	98.72%	97.56%	97.46%	97.46%	97.46%	98.60%	98.37%	98.83%	97.56%	97.56%	89.23%	98.60%	98.48%
LIO12_Maralal_Male_9yrs	99.30%	99.65%	98.84%	99.65%	99.42%	99.30%	99.77%	98.95%	99.88%	98.83%	99.53%	98.14%	98.03%	98.03%	98.03%	99.19%	99.65%	99.65%	98.14%	99.07%	90.25%	99.42%	99.53%
LIO10_Kitenpela_Male	98.95%	99.30%	98.49%	99.30%	99.07%	98.95%	99.18%	98.60%	99.07%	98.48%	99.18%	97.79%	97.69%	97.69%	97.69%	98.84%	98.83%	99.30%	97.79%	98.26%	89.46%	99.07%	98.95%
LIO9_Maralal_Female_10yrs	98.14%	98.26%	97.45%	98.26%	98.03%	98.14%	98.37%	97.80%	98.49%	97.44%	98.14%	96.76%	96.66%	96.66%	96.66%	97.80%	98.49%	98.26%	96.76%	97.69%	89.16%	98.03%	98.14%
LIO88_Amboseli_Female_1:...	98.61%	98.96%	98.15%	98.96%	98.73%	98.61%	99.07%	98.26%	99.19%	98.15%	98.84%	97.46%	97.36%	97.36%	97.36%	98.50%	98.96%	98.96%	97.46%	98.39%	89.64%	98.73%	98.84%
LIO8_Amboseli_Female_12Yrs	98.37%	98.72%	97.91%	98.72%	98.48%	98.37%	98.60%	98.02%	98.72%	97.90%	98.60%	97.21%	97.11%	97.11%	97.11%	98.26%	98.48%	98.72%	97.21%	97.68%	89.23%	98.48%	98.60%
LIO6_Lewa_Male	82.85%	82.97%	82.26%	82.97%	82.74%	82.97%	83.08%	82.76%	82.97%	82.62%	82.85%	81.75%	81.53%	81.53%	81.53%	82.55%	82.97%	82.97%	81.75%	82.25%	76.93%	82.74%	83.08%
LIO1_Maralal_Male_6Yrs	86.01%	86.01%	85.24%	86.01%	86.01%	85.91%	85.90%	85.68%	86.24%	85.42%	85.90%	84.68%	84.88%	84.88%	84.88%	85.58%	86.01%	86.01%	84.68%	85.50%	79.50%	85.78%	86.01%

LI087_C...	LI086_C...	LI076_C...	LI075_C...	LI073_C...	LI072_C...	LI071_Ol...	LI064_E...	LI061_D...	LI059_E...	LI057_E...	LI055_M...	LI054_E...	LI053_E...	LI051_E...	LI049_M...	LI048_E...	LI045_M...	LI043_M...	LI041_E...	LI039_E...	LI038_D...	LI032_S...	LI030_M...	LI029_W...
99.42%	99.65%	98.95%	98.14%	99.65%	98.48%	99.65%	98.14%	99.42%	99.77%	99.30%	99.07%	99.30%	99.18%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.53%	98.95%	95.26%	94.91%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
98.95%	99.19%	98.49%	97.90%	99.19%	98.02%	99.19%	98.60%	99.19%	98.84%	98.84%	98.84%	99.07%	98.72%	98.60%	98.61%	99.19%	99.19%	99.19%	98.60%	99.07%	98.84%	99.42%	94.91%	94.57%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
99.53%	99.77%	99.07%	98.72%	99.77%	99.07%	99.77%	98.26%	99.53%	99.42%	99.42%	99.18%	99.42%	99.30%	98.26%	99.19%	99.77%	99.77%	99.77%	98.26%	99.65%	99.42%	99.07%	95.26%	94.91%
99.42%	99.65%	98.95%	98.60%	99.65%	98.95%	99.65%	98.14%	99.42%	99.30%	99.30%	99.07%	99.30%	99.18%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.30%	98.95%	95.15%	94.80%
99.65%	99.88%	99.42%	98.60%	99.88%	98.95%	99.88%	98.37%	99.65%	99.53%	99.53%	99.30%	99.53%	99.65%	98.37%	99.30%	99.88%	99.88%	99.88%	98.37%	99.77%	99.53%	99.19%	95.38%	95.03%
99.07%	99.30%	98.60%	97.79%	99.30%	98.14%	99.30%	97.79%	99.07%	99.42%	98.95%	98.72%	98.95%	98.83%	97.79%	98.72%	99.30%	99.30%	99.30%	97.79%	99.19%	99.19%	98.61%	94.92%	94.57%
99.53%	99.77%	99.53%	98.72%	99.77%	99.07%	99.77%	98.26%	99.53%	99.42%	99.42%	99.18%	99.42%	99.77%	98.26%	99.19%	99.77%	99.77%	99.77%	98.26%	99.65%	99.42%	99.07%	95.26%	94.91%
98.95%	99.18%	98.48%	97.67%	99.18%	98.02%	99.18%	98.49%	98.95%	99.07%	98.83%	98.60%	98.95%	98.72%	98.49%	98.61%	99.18%	99.18%	99.18%	98.49%	99.07%	99.07%	98.49%	94.91%	94.57%
99.65%	99.88%	99.18%	98.37%	99.88%	98.72%	99.88%	98.60%	99.88%	99.53%	99.53%	99.53%	99.77%	99.42%	98.60%	99.30%	99.88%	99.88%	99.88%	98.60%	99.77%	99.53%	99.19%	95.38%	95.03%
98.49%	98.49%	97.79%	97.44%	98.49%	97.33%	98.49%	100%	98.49%	98.14%	98.14%	98.37%	98.84%	98.02%	100%	97.92%	98.49%	98.49%	98.49%	100%	98.37%	98.14%	98.84%	94.23%	93.88%
98.38%	98.38%	97.69%	97.57%	98.38%	97.23%	98.38%	97.58%	98.15%	98.27%	98.03%	97.80%	98.27%	97.92%	97.58%	97.81%	98.38%	98.38%	98.38%	97.58%	98.27%	98.04%	97.92%	94.37%	94.03%
98.38%	98.38%	97.69%	97.57%	98.38%	97.23%	98.38%	97.58%	98.15%	98.27%	98.03%	97.80%	98.27%	97.92%	97.58%	97.81%	98.38%	98.38%	98.38%	97.58%	98.27%	98.04%	97.92%	94.37%	94.03%
98.38%	98.38%	97.69%	97.57%	98.38%	97.23%	98.38%	97.58%	98.15%	98.27%	98.03%	97.80%	98.27%	97.92%	97.58%	97.81%	98.38%	98.38%	98.38%	97.58%	98.27%	98.04%	97.92%	94.37%	94.03%
99.53%	99.53%	98.84%	98.72%	99.53%	98.37%	99.53%	98.49%	99.30%	99.42%	99.19%	98.95%	99.19%	99.07%	98.49%	98.96%	99.53%	99.53%	99.53%	98.49%	99.42%	99.19%	99.07%	95.49%	95.14%
99.30%	99.53%	99.53%	98.72%	99.53%	99.07%	99.53%	98.02%	99.30%	99.18%	99.18%	98.95%	99.18%	99.53%	98.02%	98.95%	99.53%	99.53%	99.53%	98.02%	99.42%	99.19%	98.84%	95.03%	94.68%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
98.26%	98.49%	97.79%	97.21%	98.49%	97.33%	98.49%	99.53%	98.49%	98.14%	98.14%	98.14%	98.84%	98.02%	99.53%	97.92%	98.49%	98.49%	98.49%	99.53%	98.37%	98.14%	98.84%	94.23%	93.88%
98.49%	98.72%	98.95%	98.15%	98.72%	98.49%	98.72%	97.22%	98.49%	98.37%	98.37%	98.14%	98.37%	99.19%	97.22%	99.07%	98.72%	98.72%	98.72%	97.22%	98.61%	98.38%	98.03%	94.91%	94.57%
90.14%	90.14%	90.02%	89.71%	90.14%	90.02%	90.14%	88.93%	89.91%	90.48%	89.80%	89.57%	89.80%	90.14%	88.93%	89.62%	90.14%	90.14%	90.14%	88.93%	90.03%	90.05%	89.72%	87.08%	86.63%
99.53%	99.77%	99.07%	98.25%	99.77%	98.60%	99.77%	98.26%	99.77%	99.42%	99.42%	99.42%	99.42%	99.30%	98.26%	99.19%	99.77%	99.77%	99.77%	98.26%	99.65%	99.42%	99.07%	95.26%	94.91%
99.42%	99.65%	99.18%	98.37%	99.65%	98.72%	99.65%	98.14%	99.42%	99.77%	99.30%	99.07%	99.30%	99.42%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.53%	98.95%	95.14%	94.80%
	99.77%	99.07%	98.49%	99.77%	98.60%	99.77%	98.49%	99.53%	99.42%	99.42%	99.18%	99.42%	99.30%	98.49%	99.19%	99.77%	99.77%	99.77%	98.49%	99.65%	99.42%	99.07%	95.49%	95.14%
99.77%		99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
99.07%	99.30%		99.19%	99.30%	99.53%	99.30%	97.79%	99.07%	98.95%	98.95%	98.72%	98.95%	99.53%	97.79%	98.72%	99.30%	99.30%	99.30%	97.79%	99.18%	98.95%	98.60%	95.03%	94.45%
98.49%	98.49%	99.19%		98.49%	99.65%	98.49%	97.44%	98.25%	98.14%	98.14%	97.90%	98.14%	98.72%	97.44%	97.91%	98.49%	98.49%	98.49%	97.44%	98.37%	98.14%	98.02%	94.68%	94.10%
99.77%	100%	99.30%	98.49%		98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
98.60%	98.83%	99.53%	99.65%	98.83%		98.83%	97.33%	98.60%	98.48%	98.48%	98.25%	98.48%	99.07%	97.33%	98.26%	98.83%	98.83%	98.83%	97.33%	98.72%	98.49%	98.14%	94.57%	93.99%
99.77%	100%	99.30%	98.49%	100%	98.83%		98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
98.49%	98.49%	97.79%	97.44%	98.49%	97.33%	98.49%		98.49%	98.14%	98.14%	98.37%	98.84%	98.02%	100%	97.92%	98.49%	98.49%	98.49%	100%	98.37%	98.14%	98.84%	94.23%	93.88%
99.53%	99.77%	99.07%	98.25%	99.77%	98.60%	99.77%	98.49%		99.42%	99.42%	99.65%	99.65%	99.30%	98.49%	99.19%	99.77%	99.77%	99.77%	98.49%	99.65%	99.42%	99.07%	95.26%	94.91%
99.42%	99.65%	98.95%	98.14%	99.65%	98.48%	99.65%	98.14%	99.42%		99.30%	99.07%	99.30%	99.18%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.53%	98.95%	95.14%	94.80%
99.42%	99.65%	98.95%	98.14%	99.65%	98.48%	99.65%	98.14%	99.42%	99.30%		99.07%	99.30%	99.18%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.30%	98.95%	95.14%	94.80%
99.18%	99.42%	98.72%	97.90%	99.42%	98.25%	99.42%	98.37%	99.65%	99.07%	99.07%		99.30%	98.95%	98.37%	99.07%	99.42%	99.42%	99.42%	98.37%	99.30%	99.07%	98.95%	94.91%	94.57%
99.42%	99.65%	98.95%	98.14%	99.65%	98.48%	99.65%	98.84%	99.65%	99.30%	99.30%	99.30%		99.18%	98.84%	99.07%	99.65%	99.65%	99.65%	98.84%	99.53%	99.30%	99.19%	95.14%	94.80%
99.30%	99.53%	99.53%	98.72%	99.53%	99.07%	99.53%	98.02%	99.30%	99.18%	99.18%	98.95%	99.18%		98.02%	98.95%	99.53%	99.53%	99.53%	98.02%	99.42%	99.19%	98.84%	95.03%	94.68%
98.49%	98.49%	97.79%	97.44%	98.49%	97.33%	98.49%	100%	98.49%	98.14%	98.14%	98.37%	98.84%	98.02%		97.92%	98.49%	98.49%	98.49%	100%	98.37%	98.14%	98.84%	94.23%	93.88%
99.19%	99.42%	98.72%	97.91%	99.42%	98.26%	99.42%	97.92%	99.19%	99.07%	99.07%	99.07%	99.07%	98.95%	97.92%		99.42%	99.42%	99.42%	97.92%	99.30%	99.07%	98.96%	95.61%	95.26%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%		100%	100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%		100%	98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
99.77%	100%	99.30%	98.49%	100%	98.83%	100%	98.49%	99.77%	99.65%	99.65%	99.42%	99.65%	99.53%	98.49%	99.42%	100%	100%		98.49%	99.88%	99.65%	99.30%	95.49%	95.14%
98.49%	98.49%	97.79%	97.44%	98.49%	97.33%	98.49%	100%	98.49%	98.14%	98.14%	98.37%	98.84%	98.02%	100%	97.92%	98.49%	98.49%	98.49%		98.37%	98.14%	98.84%	94.23%	93.88%
99.65%	99.88%	99.18%	98.37%	99.88%	98.72%	99.88%	98.37%	99.65%	99.53%	99.53%	99.30%	99.53%	99.42%	98.37%	99.30%	99.88%	99.88%	99.88%	98.37%		99.53%	99.19%	95.38%	95.03%
99.42%	99.65%	98.95%	98.14%	99.65%	98.49%	99.65%	98.14%	99.42%	99.53%	99.30%	99.07%	99.30%	99.19%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%		98.96%	95.16%	94.81%
99.07%	99.30%	98.60%	98.02%	99.30%	98.14%	99.30%	98.84%	99.07%	98.95%	98.95%	98.95%	99.19%	98.84%	98.84%	98.96%	99.30%	99.30%	99.30%	98.84%	99.19%	98.96%		95.03%	94.69%
95.49%	95.49%	95.03%	94.68%	95.49%	94.57%	95.49%	94.23%	95.26%	95.14%	95.14%	94.91%	95.14%	95.03%	94.23%	95.61%	95.49%	95.49%	95.49%	94.23%	95.38%	95.16%	95.03%		99.42%
95.14%	95.14%	94.45%	94.10%	95.14%	93.99%	95.14%	93.88%	94.91%	94.80%	94.80%	94.57%	94.80%	94.68%	93.88%	95.26%	95.14%	95.14%	95.14%	93.88%	95.03%	94.81%	94.69%	99.42%	
98.60%	98.83%	98.13%	97.32%	98.83%	97.67%	98.83%	97.56%	98.60%	98.72%	98.48%	98.48%	98.72%	98.37%	97.56%	98.49%	98.83%	98.83%	98.83%	97.56%	98.72%	98.49%	98.60%	94.34%	93.99%
99.42%	99.65%	99.65%	98.84%	99.65%	99.18%	99.65%	98.14%	99.42%	99.30%	99.30%	99.07%	99.30%	99.88%	98.14%	99.07%	99.65%	99.65%	99.65%	98.14%	99.53%	99.30%	98.95%	95.14%	94.80%
99.07%	99.30%	98.83%	98.02%	99.30%	98.37%	99.30%	97.79%	99.07%	98.95%	98.95%	98.72%	98.95%	98.83%	97.79%	98.72%	99.30%	99.30%	99.30%	97.79%	99.18%	98.95%	98.60%	94.80%	94.45%
98.03%	98.26%	98.49%	97.91%	98.26%	98.26%	98.26%	96.76%	98.03%	97.91%	97.91%	97.68%	97.91%	98.49%	96.76%	97.69%	98.26%	98.26%	98.26%	96.76%	98.14%	97.91%	97.57%	94.48%	94.13%
98.73%	98.96%	98.96%	98.15%	98.96%	98.49%	98.96%	97.46%	98.73%	98.61%	98.61%	98.38%	98.61%	99.19%	97.46%	98.39%	98.96%	98.96%	98.96%	97.46%	98.84%	98.61%	98.27%	94.49%	94.14%
98.48%	98.72%	98.25%	97.44%	98.72%	97.79%	98.72%	97.21%	98.48%	98.37%	98.37%	98.14%	98.37%	98.48%	97.21%	98.14%	98.72%	98.72%	98.72%	97.21%	98.60%	98.37%	98.03%	94.23%	93.88%
82.74%	82.97%	82.97%	82.43%	82.97%	82.74%	82.97%	81.75%	82.74%	82.85%	82.62%	82.39%	82.62%	82.97%	81.75%	82.47%	82.97%	82.97%	82.97%	81.75%	82.87%	82.66%	82.43%	80.46%	80.34%
86.01%	86.01%	85.78%	85.24%	86.01%	85.55%	86.01%	84.68%	85.78%	85.78%	85.90%	85.43%	85.78%	86.24%	84.68%	85.50%	86.01%	86.01%	86.01%	84.68%	85.91%	85.70%	85.37%	84.08%	84.19%

LIO16_S...	LIO12_M...	LIO10_Kl...	LIO9_Ma...	LIO8B_A...	LIO8_Am...	LIO6_Le...	LIO1_Ma...
98.48%	99.30%	98.95%	98.14%	98.61%	98.37%	82.85%	86.01%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
98.25%	98.84%	98.49%	97.45%	98.15%	97.91%	82.20%	85.24%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
98.60%	99.42%	99.07%	98.03%	98.73%	98.48%	82.74%	86.01%
98.48%	99.30%	98.95%	98.14%	98.61%	98.37%	82.97%	85.91%
98.72%	99.77%	99.18%	98.37%	99.07%	98.60%	83.08%	85.90%
98.14%	98.95%	98.60%	97.80%	98.26%	98.02%	82.76%	85.68%
98.60%	99.88%	99.07%	98.49%	99.19%	98.72%	82.97%	86.24%
98.02%	98.83%	98.48%	97.44%	98.15%	97.90%	82.62%	85.42%
98.72%	99.53%	99.18%	98.14%	98.84%	98.60%	82.85%	85.90%
97.56%	98.14%	97.79%	96.76%	97.46%	97.21%	81.75%	84.68%
97.46%	98.03%	97.69%	96.66%	97.36%	97.11%	81.53%	84.88%
97.46%	98.03%	97.69%	96.66%	97.36%	97.11%	81.53%	84.88%
97.46%	98.03%	97.69%	96.66%	97.36%	97.11%	81.53%	84.88%
98.60%	99.19%	98.84%	97.80%	98.50%	98.26%	82.55%	85.58%
98.37%	99.65%	98.83%	98.49%	98.96%	98.48%	82.97%	86.01%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
97.56%	98.14%	97.79%	96.76%	97.46%	97.21%	81.75%	84.68%
97.56%	99.07%	98.26%	97.69%	98.39%	97.68%	82.25%	85.50%
89.23%	90.25%	89.46%	89.16%	89.64%	89.23%	76.93%	79.50%
98.60%	99.42%	99.07%	98.03%	98.73%	98.48%	82.74%	85.78%
98.48%	99.53%	98.95%	98.14%	98.84%	98.60%	83.08%	86.01%
98.60%	99.42%	99.07%	98.03%	98.73%	98.48%	82.74%	86.01%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
98.13%	99.65%	98.83%	98.49%	98.96%	98.25%	82.97%	85.78%
97.32%	98.84%	98.02%	97.91%	98.15%	97.44%	82.43%	85.24%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
97.67%	99.18%	98.37%	98.26%	98.49%	97.79%	82.74%	85.55%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
97.56%	98.14%	97.79%	96.76%	97.46%	97.21%	81.75%	84.68%
98.60%	99.42%	99.07%	98.03%	98.73%	98.48%	82.74%	85.78%
98.72%	99.30%	98.95%	97.91%	98.61%	98.37%	82.85%	85.78%
98.48%	99.30%	98.95%	97.91%	98.61%	98.37%	82.62%	85.90%
98.48%	99.07%	98.72%	97.68%	98.38%	98.14%	82.39%	85.43%
98.72%	99.30%	98.95%	97.91%	98.61%	98.37%	82.62%	85.78%
98.37%	99.88%	98.83%	98.49%	99.19%	98.48%	82.97%	86.24%
97.56%	98.14%	97.79%	96.76%	97.46%	97.21%	81.75%	84.68%
98.49%	99.07%	98.72%	97.69%	98.39%	98.14%	82.47%	85.50%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
98.83%	99.65%	99.30%	98.26%	98.96%	98.72%	82.97%	86.01%
97.56%	98.14%	97.79%	96.76%	97.46%	97.21%	81.75%	84.68%
98.72%	99.53%	99.18%	98.14%	98.84%	98.60%	82.87%	85.91%
98.49%	99.30%	98.95%	97.91%	98.61%	98.37%	82.66%	85.70%
98.60%	98.95%	98.60%	97.57%	98.27%	98.03%	82.43%	85.37%
94.34%	95.14%	94.80%	94.48%	94.49%	94.23%	80.46%	84.08%
93.99%	94.80%	94.45%	94.13%	94.14%	93.88%	80.34%	84.19%
	98.48%	98.37%	97.10%	97.80%	98.72%	81.82%	85.20%
98.48%		98.95%	98.61%	99.30%	98.60%	83.08%	86.13%
98.37%	98.95%		97.56%	98.26%	98.48%	82.28%	85.55%
97.10%	98.61%	97.56%		97.92%	97.22%	82.47%	86.59%
97.80%	99.30%	98.26%	97.92%		97.92%	82.51%	85.53%
98.72%	98.60%	98.48%	97.22%	97.92%		81.95%	85.20%
81.82%	83.08%	82.28%	82.47%	82.51%	81.95%		71.61%
85.20%	86.13%	85.55%	86.59%	85.53%	85.20%	71.61%	