# POPULATION SIZE AND GENETIC DIVERSITY OF NIGERIAN LIONS *(PANTHERA LEO)*

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Abstract

High growth rate in human populations, agricultural developments and industrialization have impinged negatively on the natural habitats of most large carnivores, causing fragmentation, isolation and consequently reduction in population size. Lions (*Panthera leo*) that once roamed most parts of Southern Europe, Asia, the Middle East, North America, northern part of South America and most parts of Africa are today found only in parts of sub-Saharan Africa and at a single site in India. Even in these areas, they are increasingly confined to supposedly protected areas where their numbers are gradually decreasing. In West Africa, lions are found only in national parks, game reserves and zoological gardens. In Nigeria, the only areas known to still have wild lions are Yankari Game Reserve (YGR), in Central North East and Kainji-Lake National Park (KLNP), in the West. I investigated the trend of events into the decline of lions by reviewing lion articles as far back as possible with the aim of understanding variables responsible for their decline over the years. I employed the method of non-invasive sampling of faeces in this study to gather information about the population size of lions in Nigeria. However, studies that employ non-invasive sampling are often confronted with pitfalls due to degraded DNA and non-uniform distribution of sloughed intestinal cells of the focal species in the faeces, which can result in low genotyping success. It is therefore important to store samples in conditions that can preserve the minute DNA quantity in the faeces at the time of collection prior to laboratory analysis. I investigated which medium may be most appropriate for storage of lion faecal DNA, and I found that amplification success was highest for samples preserved in ethanol. I investigated the pattern of phylogeographic distribution of the Nigerian lions and lions in other parts of Africa and India. The aim was to understand the genetic make-up of the Nigerian lion within the West and Central African range so as to aid in future conservation and management decisions. I found that the two lion populations in Nigeria have different origins. Lions in YGR resemble more closely Cameroon lions while the population in KLNP resembles closely the lion population in Benin. Furthermore, I conducted a pilot study in YGR using lion faecal samples collected opportunistically along game viewing and patrol tracks to test the feasibility and reliability of obtaining DNA from faeces collected from a tropical environment to identify individuals. This method proved feasible as eleven individuals were identified from two polymorphic microsatellite loci. The feasibility of the pilot survey prompted the extension of the study to KLNP with the aim to estimate the population size and the level of gene flow that may exist between these two lion populations. I found that the number of individuals within YGR and KLNP are small and there was no evidence for gene flow between them. To secure the long-term persistence of wild lions in Nigeria will require immediate and concerted actions from state, national and regional stakeholders.



Keywords: non-invasive sampling, faecal DNA, protected area, isolated population, *Panthera leo*

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A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted or in ms).



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# LIST OF PAPERS

This thesis is based on the following papers which are referred to by their Roman numerals.

I Ulf Ottosson, Talatu Tende, Christian Hjort & Bengt Hansson. Populations and geographical distribution of Lions (*Panthera leo*) in West Africa from ancient to present times, especially during the last 200 years.
- *Submitted*

II Talatu Tende, Bengt Hansson, Ulf Ottosson, Staffan Bensch. 2014. Evaluating preservation medium for the storage of DNA in the African lion (*Panthera leo*) faecal samples.
- *Current zoology* in press

III Talatu Tende, Staffan Bensch, Ulf Ottosson, Bengt Hansson. Dual phylogenetic origins of Nigerian lions (*Panthera leo*).
- *Submitted*

IV Talatu Tende, Ulf Ottosson, Bengt Hansson, Mikael Åkesson, Staffan Bensch. 2010. Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling.
- *African Journal of Ecology* 48:949-952

V Talatu Tende, Bengt Hansson, Ulf Ottosson, Mikael Åkesson, Staffan Bensch. 2013. Individual Identification and genetic variation of lions (*Panthera leo*) from two protected areas in Nigeria.
- *Plos One* in press

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# LIST OF CONTRIBUTIONS

**Paper I.** The idea was formulated by Ulf Ottosson (UO), Talatu Tende (TT) and Bengt Hansson (BH). UO and Christian Hjort (CH) analysed the data and wrote the manuscript with support from co-authors.

**Paper II.** The study was planned by TT and UO. Sample collection was organised by TT and UO. TT analysed the data, and wrote the manuscript with support from other co-authors.

**Paper III.** The idea was formulated by all co-authors. TT carried out the analyses with support from BH and Staffan Bensch (SB). TT wrote the manuscript with support from all co-authors.

**Paper IV.** The study was conceived by TT and UO. Field data collection was organised by TT and UO. Laboratory work was performed by TT. Analyses were done by TT, BH and Mikael Åkesson (MÅ). TT wrote the manuscript with support from co-authors.

**Paper V.** TT and UO conceived the study. Field data collection was organised by TT and UO. Laboratory work was performed by TT, MÅ & BH. All analyses were done by TT with assistance from BH and SB. TT wrote the manuscript with support from other co-authors.

# POPULATION SIZE AND GENETIC DIVERSITY OF NIGERIAN LIONS *(PANTHERA LEO)*

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## INTRODUCTION

High growth rate in human populations, agricultural developments and industrialization are leading increasingly to biodiversity loss (Ceballos & Ehrlich 2002, Estes *et al.* 2011). These anthropogenic activities are responsible for the loss of natural habitats, habitat fragmentation and isolation of natural populations. This is driving many species to exist in only small-sized populations. Once a continuous population has been fragmented into smaller patches with reduction in size, isolation from other populations with restriction in movement, random demographic and genetic processes can lead the population rapidly towards extinction (Wilcox & Murphy 1985, Nowell & Jackson 1996, Turner 1996 in Callens *et al.* 2011).

Large carnivores have suffered great reductions in their populations and range sizes over the past 50 years mostly due to anthropogenic activities (Nowell & Jackson 1996, Estes *et al.* 2011, Riggio *et al.* 2012). Their requirements for large home range, limited dispersal ability, low reproductive rates and subsequent low densities have made them exceptionally sensitive to habitat changes (Proctor *et al.* 2005, Howe *et al.* 2006). If small isolated populations are vulnerable to extinctions, then large carnivores are especially prone to extinction due to the aforementioned reasons (Woodroffe & Grinsberg, 1998). Many studies have been carried out on carnivores which revealed that most populations are now declining (Craigie *et al.* 2010) and even the remnant populations exist in small, fragmented isolates (Riggio *et al.* 2012). For instance a study by Proctor *et al.* (2005) in the United States of America on grizzly bears (*Ursus arctos* ssp.) revealed a range contraction and that the remaining populations exist in only four fragmented isolates in vulnerably small number (≤100 individuals). The Arabian leopards (*Panthera pardus nimr*) in Israel have also faced a severe reduction in their population size due to anthropogenic activities (Perez *et al.* 2006). This population is predicted to be at the brink of extinction. Also the wild Scandinavian wolf (*Canis lupus*) experienced extinction during the first half of the 20th century before re-establishment by immigrants from Finland/ Russia in the beginning of 1980s (Liberg *et al.* 2005, Bensch *et al.* 2006).

The lions were once known to roam most parts of Africa, Southern Europe, the Middle East and Asia (Bauer & Merwe 2004, Nowell & Jackson 1996). Today they are only found

in sub-Saharan Africa and at one locality in India, where they are being increasingly restricted to supposedly protected areas and often in declining numbers (Nowell & Jackson 1996, Chardonnet 2002, Bauer & Merwe 2004, Wilson & Mittermeier 2009). A recent survey has shown that the African lions are in rapid decline due to habitat loss, with a population decrease from around 100,000 animals just fifty years ago to as few as 32,000 today (Packer *et al*. 2013). Alarmingly, lions have been predicted to be at the verge of extinction in the near future, just 20-40 years from now (Nowell & Jackson 1996, Bauer & Merwe 2004, Riggio *et al*. 2012, Packer *et al*. 2013).

In West Africa lions are found only in protected areas such as national parks, game reserves and zoological gardens. In Nigeria today only two protected areas still have wild lions; Yankari Game Reserve in Central North East and Kainji-Lake National Park in the West.

When the population of any species becomes reduced in size and gets isolated for a long period of time it brings about erosion of genetic diversity. This compromises the evolutionary potential of such population to environmental changes (Falconer & Mackay 1996, Frankham 1995, 1996, Keller & Waller 2002). Also such small populations are often faced with a higher risk of extinction from environmental catastrophes (Saccheri *et al*. 1998, Dunham *et al.* 1999, Bijlsma *et al*. 2000, Higgins & Lynch 2001) than large interconnected populations (Lande 1993, Frankham 1996, Lacy 2000). The smaller the population, the more susceptible it is to chance events. The influence of stochastic variation in demographic (reproduction and mortality) rates is higher for small populations than large ones, causing random fluctuation in size and a greater probability that it will lead to extinction. Also in small populations mating is prone to occur between individuals that are closely related genetically (inbreeding). Mating between close relatives i.e. inbreeding results in recessive deleterious alleles coming into the limelight as homozygous in the offspring. This increases the chances of offspring being affected by harmful effects, and indeed inbreeding is known to cause deleterious effects on all aspects of reproduction and survival (Frankham *et al*. 2002, Keller & Waller 2002, Bjorklund 2003). The negative consequences of inbreeding on fitness is called inbreeding depression. It can be expressed as reduction in clutch size (in the case of birds), fewer or smaller cubs, lower cub survival (in the case of mammals), and physiological defects as well as stillborn. Loss of genetic variation due to genetic drift and inbreeding depression is assumed to be a serious problem for the conservation of small populations (Gilpin & Soule 1986). All species experience environmental change whether it is climate change, altered competition, new diseases or new predators (Frankham & Kingslover, 2004). Genetic diversity is the raw material needed in order to evolve the ability to cope with the aforementioned environmental challenges. When the genetic diversity is high it is more likely that some individuals within a population will possess the alleles suited to cope with the environmental change. There are many studies on lions demonstrating the harmful effects of inbreeding. During the period of 2003 and 2008 in a zoological garden in northern Italy, 19 white lions were recorded to be born to parents that were half-sibs (Scaglione *et al.* 2010). Out of these 19 newborns, four were stillborns, 13 died within one month and one was artificially fed up to six months and then died. Further investigations into these individuals revealed congenital anomalies and cranial malfunctions known as Arnold-Chiari malfunction. The cranial malfunctions detected in offspring of the parents that were half-sib points to negative effects of inbreeding. White lions (*P. leo krugeri*) were also recorded in South Africa between 1928 and 1940s in the Timbavati

and Kruger National Park regions (McBride 1977), although it is not known whether they resulted from inbreeding. Packer *et al.* (1991) found a decline in reproductive performance with increased inbreeding in the lion populations in the Ngorongoro Crater in Tanzania. At the same locality, Brown *et al.* (1991) found reduced sperm production in the ejaculates of lions in the population. A study carried out by Munso *et al.* (1996) some years later found that these inbred Ngorongoro lions had lower spermatid levels with fewer seminiferous tubular areas per testis which could be responsible for the low sperm production that was recorded in the study by Brown *et al*. (1991). A study carried out by Wildt *et al.* (1987) has associated low genetic diversity with poor seminal quality in lions. Moreover, inbreeding has caused several measurable reductions in reproductive rates and disease resistance in several other small lion populations (Kissui & Packer 2004, Trinkel *et al*. 2008, 2011).

For proper conservation and management of remnant wildlife adequate information on population size, connectivity between fragments of populations and genetic health are very important (Creel *et al.* 2003). As most natural areas become smaller and more fragmented by the day, it is therefore important to understand the ecological and evolutionary dynamics of these small populations for effective management (Lande 1988).

**Sampling strategies for rare, cryptic and/or endangered species**

Lions just like other large terrestrial carnivores are usually very difficult to count. This is due to their low population densities, elusive behaviour as well as their ability to cover large home ranges. An effort to conduct complete counts of a lion population is thus likely to be both organizationally difficult and time consuming (Balme *et al.* 2007). The alternative is to interpolate population sizes using different sampling strategies.

Over the years genetic approaches have played important roles in assisting to answer ecological questions such as estimating or monitoring the population size of a species, determining the home ranges of individuals in a population as well as determining the genetic status of populations. The employment of non-invasive samples using improved genetic methods has gained increasing importance in conservation biology especially when studying wild populations of rare, cryptic or endangered species (Bhagavatula & Singh 2006, Schwartz *et al.* 2007). Non-invasive sampling is the collection of animal remains for population studies without having to trap or even directly observe the animals under study (Taberlet *et al*. 1997, Taberlet & Luikart 1999). This approach obviously gives access to more samples and also an additional advantage of reducing the possible amount of harm inflicted on the animals (De Barb *et al.* 2010). Because faecal samples can be obtained without capturing the individual species under study this method has great promise for population estimates (Creel *et al.* 2003) and genetic studies of endangered species. Today, samples such as faeces, shed feathers, shed skin, hairs, scales and urine can provide a ready source of template DNA to be used in PCR for population estimates and genetic studies (Briker *et al.*1996; Morin & Woodruff 1996; Valsecchi *et al.* 1998, Rudnick *et al.* 2005).

DNA-based analysis of faeces (Höss *et al.* 1992) is a potentially reliable method for estimating population sizes and it suits well for large carnivores. This is because faecal samples are easily encountered and recovered along trails where they defecate to mark territory boundaries (Kohn & Wayne 1997, Kohn *et al.* 1999, Macdonald 1983).

Various methods have been developed to enhance the extraction of DNA from sources such as hair, shed skin, feathers

and faeces (Taberlet *et al.* 1996, Gagneux *et al.* 1997, Goossens *et al.* 1998, Kohn *et al.* 1999). These extracted DNA samples can then be PCR amplified and used to genotype individuals through the count of distinct microsatellite genotypes at multiple loci (Creel *et al.* 2003) thus promoting the use of DNA from noninvasive samples to estimate population size.

Many studies have employed the method of non-invasive genetic sampling to either estimate population size or to monitor populations of different carnivore species (Taberlet *et al.* 1997, Piggott & Taylor 2003, Waits & Paetkau 2005, Schwartz *et al.* 2007). By studying appropriate nuclear markers (most often microsatellites), analysis of non-invasive genetic samples (e.g. faeces) collected opportunistically from the field can provide individual identification, home range, adequate information on population size, sex identification as well as genetic status within and between populations (Taberlet *et al.* 1997). This method has been successfully used in studies on e.g. mountain lions (*Puma color*) in California (Ernest *et al.* 2000), wolves (*Canis lupus*) in Scandinavia (Bensch *et al.* 2006), brown bears (*Ursus arctos*) in central Austria (Kruckenhauser *et al.* 2009) and the United States of America (Poole *et al.* 2001, Boulanger *et al.* 2004, Paetkau 2003), tiger (*P. tigris*) in India (Mondol *et al.* 2009) and the snow leopard (*P. uncia*) in central Asia (Waits *et al.* 2006).

Although the use of non-invasive sampling technique is promising for population studies, there are a number of pitfalls that are associated with it. Previous studies have observed errors like amplification failure, allelic drop-out and false alleles in microsatellite analyses which can significantly affect population size estimates (Taberlet *et al.* 1997, Mills *et al.* 2000, Waits *et al.* 2001, Creel *et al.* 2003, McKelvey & Schwartz 2004). These errors are due to the fact that DNA extracted from non-invasive samples is often of low quantity and quality (degraded DNA; Taberlet *et al.* 1999, Wandeler *et al.* 2003, Pompano *et al.* 2005). Allelic drop-out is the result of heterozygotes being typed as homozygotes due to failure of amplification of one of the alleles. Allelic drop out may be caused by sampling stochasticity when pipetting the template DNA in a diluted extract, where sometimes only one of the two alleles is pipetted, amplified and detected (Miller & Waits 2003). That is why it is ideal to use PCR primers that amplify short DNA fragments (Taberlet *et al.* 1997, Pompanon *et al.* 2005). False alleles are allele artifacts that are often generated during PCR which are not true alleles but might be mistyped as alleles.

**Polymerase Chain Reaction (PCR) and Microsatellites**

Several years ago large amounts of fresh tissue often from several organs of a study species were needed to carry out studies employing genetic methods on wild animal populations (Lewonti 1991, Murphy *et al.* 1996) using protein electrophoresis. This required killing the animal under study (Taberlet & Luikart 1999) thereby impacting negatively on the population or species under study especially if the species or population are threatened. This context changed dramatically with the invention of the polymerase chain reaction (PCR; Saiki *et al.* 1985, Mullis 1990), which requires only minute quantity of starting material using a thermostable DNA polymerase (Mullis & Faloona 1987, Saiki *et al.* 1988). This new technique has been employed widely by population geneticists to answer ecological questions (Saiki *et al.* 1988, Wright & Wynford-Thomas 1990, Erlich *et al.* 1991). This has helped to put a stop to the destructive sampling method employed decades ago.

Microsatellites, also known as simple sequence repeats or simple tandem repeats (SSR or STR) are regions within the genome

where short motifs of DNA nucleotides are repeated (e.g. CACACACACA) ( Litt & Luty 1989, Tautz 1989. These sequence repeats can either be mono, di, tri, or tetra repeats and they are often common and evenly distributed in the genome (Ellegren 2004). The main property of microsatellites is that they have high mutation rates and therefore display high variation within populations as well as between individuals (Sunnucks 2000, Spong *et al.* 2000, Beebee & Rowe 2008). The regions surrounding the microsatellite repeat sequence – the flanking regions – are much more conserved and show less variation. These flanking regions can therefore be used as targets for primers to amplify the microsatellite loci by PCR. This process will produce enough copies of DNA, which can then be used to genotype and identify individuals and thereby estimate population size. What makes microsatellite loci interesting as a marker of choice in population studies is the variation in the number of sequence repeat units between individuals in a population and/ or between populations (Sunnucks 2000, Spong *et al.* 2000; Fig. 1). There is more variation at microsatellite loci than at other nuclear regions due to the microsatellites' high mutation rates caused by their unique mutational processes (e.g. strand slippage) (Bhargava & Fuentes 2010, Pokhriyal *et al.* 2012). By looking at differences in the number of repeat units, individuals can be identified and conclusions can be reached about population size and as well as population structure.

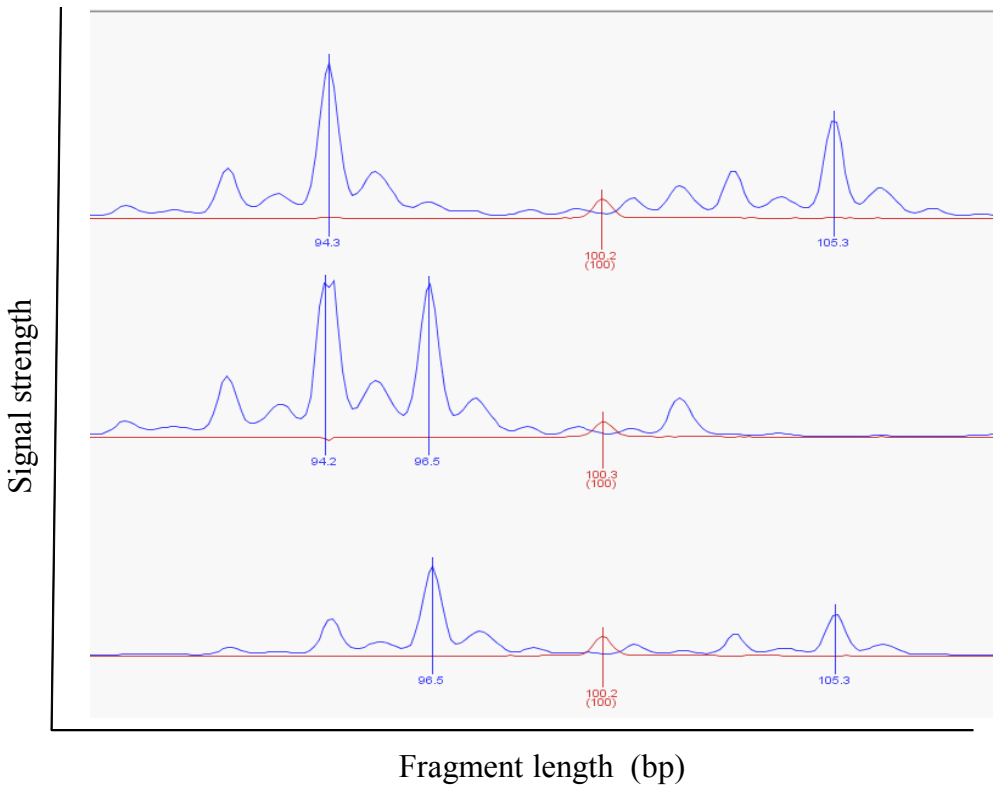


**Figure1:** Electropherogram showing alleles of three individuals at one microsatellite locus.

In inbred populations, many individuals carry identical genotypes at many loci due to the fact that genetic diversity is often low. This makes it important to choose highly variable markers when trying to differentiate between individuals and accurately estimate population size.

## AIMS OF THE THESIS

The overall aim of this study is to estimate the population size and genetic diversity within and between the wild lion populations in Nigeria using faecal sample DNA. It is important to know the size as well as the genetic status of these two populations for proper management.

This study specifically aims to:

I) Review and establish the lion's historical range in West Africa in general and in Nigeria in particular as far back as possible.

II) Determine the storage method that best preserves the minute DNA in faeces and also enhances PCR amplification success of both the mitochondrial and nuclear DNA.

III) Determine the relationship of Nigerian lions to lions in other parts of Africa and Asia.

IV) Test the feasibility and reliability of using lion faecal sample DNA to genotype and identify individuals.

V) Estimate the population size and level of gene flow that may exist within and between the wild lion populations in Nigerian.

## MATERIALS AND METHODS

### Study sites

The study was carried out in two protected areas in Nigeria: Yankari Game Reserve (YGR) and Kainji-Lake National Park (KLNP) (Fig. 2).

YGR is located in Central North-East Nigeria with a landmass of 2,244 km$^2$ (9° 50'N and 10° 30'E). The reserve lies in the Sudan Savanna zone and the vegetation is composed mainly of dry savannah woodland with a narrow floodplain, bordered by patches of gallery and riparian forests (Geerling 1973, Crick & Marshall 1981, Green 1989). Vegetation here is comprised of common woodland tree species such as *Afzelia africana, Burkea africana, Pterocarpus erinaceus, Isoberlina doka, Monotes kestingii, Combretum glutinosum, Detarium microcarpum* and *Anogeissus leiocarpus. Gardenia aqualla* and *Dischrostachis glomerata* are frequent in the shrub layer, while *Hyparrhenia involucrate* and *H. bagirmica* are the dominant grasses. YGR receives an average rainfall of about 1000 mm a year which occurs between April and October (Crick & Marshall 1981). Although during the study period rainfall was rather observed to be between May and October (Tende T, pers. Obs.).

KLNP is located in the Western part of Nigeria (09° 55'N 03° 57'E) and occupies a landmass of 5,340 km$^2$. The vegetation is made up primarily of Guinea savanna woodland. Common woodland species include *Terminalia macroptera* found along the Oli River, which flows in the centre of the Park, *Detarium microcarpum* and *Borkea africana* woodland occupy about 70% of the Park area. *Isoberlinia tomentosa* woodland play vital role in providing shelter and cover for game. The mean annual rainfall is between 1000 and 1200 mm per year and occurs between April and October, with the highest peak of rain in September (Afolayan 1978).

In paper I of this thesis we reviewed articles that have attempted to establish the status of lions in most of its range. The aim was to establish the lion's historical range as far back as possible, by describing the decrease in the lion population with references to human population, anthropogenic effects

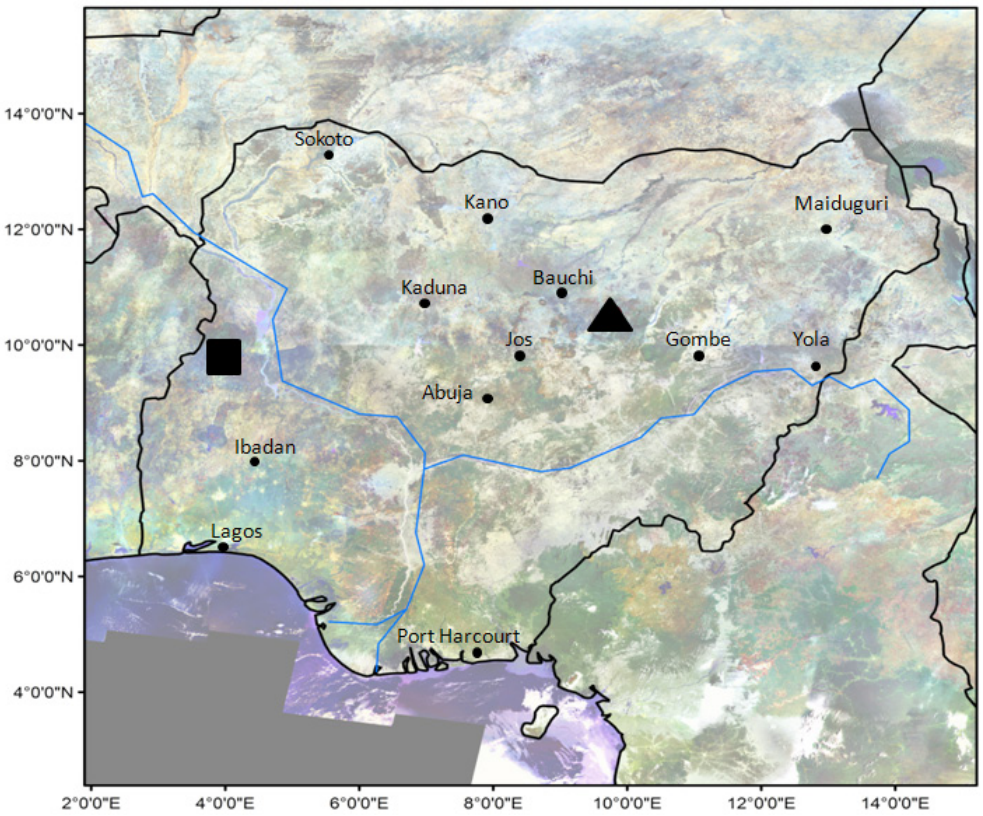


**Figure 2:** Map of Nigeria with some major cities and positions of the two survey sites; Kainji-Lake National Park (KLNP) in black square and Yankari Game Reserve (YGR) in black triangle.

and climatic events etc. This will help to shed more light on the trend of events responsible for their decline, and if possible assist on devising means to overcome these factors to save the remaining population.

Various studies have shown that the population of African lions have declined and they are found today only in sub-Saharan Africa and at one locality in India. Even in these remnant places they remain restricted to supposedly protected areas (Nowell & Jackson 1996, Chardonnet 2002, Bauer & Merwe 2004, Wilson & Mittermeier 2009). Lions in Nigeria are today confined to only two areas the Yankari Game Reserve and Kainji Lake National Park, and in low numbers. Both areas are characterized by quite dense vegetation and lions are rarely seen, but there are more sightings of their footprints, faeces.

Thus I employed the method of non-invasive sampling of lion faeces. Due to pitfalls associated with non-invasive sampling because of degraded DNA that can result in low genotyping success, I deemed it important to investigate the medium that may be most appropriate to store and preserve lion faecal DNA before laboratory analysis in paper II. In paper III, I investigated the pattern of phylogeographic distribution of the Nigerian lions and lions in other parts of Africa and India in order to understand the genetic make-up of the Nigerian lion

within the West and Central African range. In papers IV and V, I used the non-invasive sampling technique to gather information on the population size and genetic status of the two wild lion populations in Nigeria.

**Sample collection**

Lion faeces were collected opportunistically along game viewing and patrol tracks within the two study sites respectively (Figs. 2 & 3).

**Figure 3:** Sample collection in Yankari Game Reserve

A total of 3,724 hours were spent sampling in YGR during data collection between 2008 and 2012, and 294 hours were spent sampling in KLNP in 2009, 2010 and 2012. The Global Positioning System (GPS) was used to record the position of each sample collected. All samples were preserved in 95 % ethanol at room temperature (except samples for the investigation of preservation medium which were preserved in three different media). Thereafter taken to Lund University, Sweden and kept in a freezer at -40°C before DNA extraction.

The number of pitfalls known to be associated with non-invasive sampling due to degraded DNA (Taberlet *et al.* 1999, Pompano *et al*. 2005) prompted our efforts to test and employ the most appropriate medium for the storage of lion faecal DNA prior to laboratory analysis in paper II. The amount of DNA initially present in faecal samples and the amount still present after collection and storage, can determine the amount of DNA that can be available for downstream analyses. Due to the fragile nature of DNA and its low quantity in non invasive samples it is vital to preserve the minute DNA in these samples in good medium prior to analysis; especially if samples will not be analysed immediately after collection. The choice of a good preservation medium should be of prime importance for every genetic study especially when collected samples cannot be processed immediately at the collection site. The identification of effective preservation methods can enhance PCR amplification success rate, increase genotyping accuracy and also increase the feasibility of using faecal DNA for genetic studies. We collected twenty three fresh faecal samples of similar age, determined to be 1 hour and 1 week old, because we often travelled on these tracks more than once per day and at least on a weekly basis. These were preserved in three different media (ethanol, ASL buffer and Two-step storage). Faeces freshness was determined based on moisture content, appearance and strength of odor (Rutledge *et al*. 2008, Vynne *et al*. 2011). The aim of the study was to determine the field preservation method that best preserves DNA and enhances PCR amplification success of both mitochondrial and nuclear DNA.

In order to evaluate the amplification success of the mitochondrial DNA, all extracted samples were PCR amplified using a pair of primers. To evaluate amplification success of the nuclear DNA all positive samples found to originate from lion were PCR amplified using six polymorphic microsatellite primers (FCA001, FCA026, FCA031, FCA077, FCA506 and FCA567; Menotti-Raymond *et al.* 1999).

Direct and parallel comparison of the amplification success of the samples obtained from the three different media was made from the genotype result obtained from the six microsatellite markers, by direct count of the number of successful amplifications. Success rate was then estimated as the number of samples that amplified at a locus from each medium. We tested if the probability of success or failure (1, 0) at the six different loci is dependent on preservation method by building a generalized linear mixed model (GLMM) with a binomial error structure with the probability of success as the response variable, preservation method (Ethanol, ASL buffer or Two-step storage) and loci (1-6) as explanatory variables and sample replicates as a random factor, thus:

Success (1/ 0) =
preservation method (Ethanol, ASL buffer, Two-step storage) + loci (1-6) + replicates (random factor)

The model also included two-way interactions of the explanatory variables and, using stepwise backward elimination process, the final model, which best explains the variability in the data, was selected based on the value of the Akaike Information Criterion (AIC). The best-fit model was the one with

the lowest AIC value.

Historical and current decline of lion populations in most of its range necessitates an investigation into their geographic and genetic pattern of distribution as well as their extent of overlap (Barnett *et al.* 2006 & 2009). This is so as to enhance our knowledge of the various populations and shed light on the amount of efforts that need to be devoted when setting up management strategies for the remaining populations. A recent study by Bertola *et al.* (2011) has shown that lions in West and central Africa are genetically different from those in East and Southern Africa and they resemble more closely the Asiatic lion populations. Although the study by Bertola and colleagues shows a close relationship between West and Central African lions with the Asiatic lion population, their study did not incorporate lions from other parts of West Africa such as Nigeria where lions still exist. The incorporation and analysis of the Nigerian lion with lions from other parts of the range is important in order to broaden our knowledge about their geographic patterns of distribution and phylogenetic relationship and thus make future management decisions easier. To achieve this, lion faecal DNA extracts from four supposedly unrelated individuals each that have been identified from Yankari Game Reserve, Central North East and Kainji-Lake National Park, Western Nigeria were PCR amplified. Primers were designed to amplify three different segments of 1200 base pairs of the mitochondrial cytochrome *b* region (Paper III). The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining. Samples were further Sanger sequenced using the forward primers (BigDye sequencing kit; Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems). Sequences were visually checked and manually aligned using Geneious vs.5.6.6 against sixty one lion reference sequences from other parts of Africa and India downloaded from Genbank. The program MEGA5 was used to analyze the sequence data for the construction of phylogenetic trees.

To test the feasibility and reliability of non-invasive sampling in population study in tropical region, we conducted a pilot study within Yankari Game Reserve (Paper IV) following suggestions by Taberlet *et al.* (1999) before embarking on a large-scale study. This was done to evaluate how feasible and reliable it is to use lion faecal sample DNA collected from tropical regions to identify and genotype individuals. Most studies which employed the use of faecal DNA in tropical environments have been conducted on primates (Gerloff *et al*. 1995, Frantzen *et al*. 1998, Bayes *et al*. 2000, Nsubuga *et al*. 2004 Vallet *et al*. 2007) with very few studies carried out on other mammal species (Gobush *et al*. 2009, Vynne *et al*. 2011). Studies that have been conducted on other mammals have been carried out mostly in temperate environments, e.g. mountain lions in California (Ernest *et al.* 2000), wolves in Scandinavia (Bensch *et al.* 2006) and coyotes *C. latrans* in Mexico (Panasci *et al*. 2011). In this pilot study, DNA was extracted from 108 faecal samples using the stool QIAamp® DNA Stool Mini Kit (Qiagen) according to manufacturer's instructions.

Contamination of DNA during extraction or the PCR process can be a major problem when using non-invasive DNA. This was carefully taken care of by conforming to guidelines to avoid this through the use of a blank as a control during the extraction and PCR processes. Also DNA extracts from two lion tissue samples obtained from Göran Spong (Umeå, Sweden) in his project on lions in Tanzania were used as positive controls in our PCR set up in order to check the reliability of genotypes obtained from our samples.

### Species Identity

In order to differentiate the faeces of the lion from that of other possible carnivores (e.g. spotted hyena, *Crocuta crocuta*, and striped hyena, *Hyena hyena*) that might occur within the study areas, a short (206 bp) portion of the mitochondrial cytochrome *b* gene was PCR-amplified and sequenced. The primers LIHYF (5'-ATGACCAACATTCGAAAATCWC-3') and LIHYR (5'-ATGTGGGTSACTGATGAG-3') were designed to avoid amplification of human and ungulate DNA in general, in order to promote detection of the target species (Tende *et al.* 2010). A blank control (reagents only) was included in all PCRs to monitor for contamination. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining. Positive samples were further sequenced using LIHY forward primer (BigDye sequencing kit; Applied Biosystems) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems). The sequences were checked visually and aligned manually against species reference sequences (Ascension numbers; EF437586.1, AJ809332.1 and EF107524.1) obtained from Genbank to determine species identity.

### Individual Identity

Samples confirmed to be from lions were then further PCR amplified using two microsatellite loci (locus Ple53 and locus Ple56, Gaur *et al*. 2006). PCR products were separated on 6% polyacrylamide gels and alleles detected in a Typhoon 9200 (Amersham Biosciences, Piscataway, New Jersey U.S.A.). This was then scored for individual identification and genetic variability. To deal with the problem of allelic drop out that is commonly associated with using non-invasive samples due to low quality DNA, in our analyses, all samples were amplified at each locus three times for confirmation.

The feasibility and reliability of using lion faecal sample DNA in our pilot study prompted us to extend the study from YGR to the other park in Nigeria, KLNP (see Fig. 2), that still holds lions (paper V). Faecal samples were collected opportunistically along patrol and game viewing tracks in the park. The aim was to determine the population size and genetic diversity of lions within this Park. Knowledge about the population size and genetic variability of the lions in YGR and KLNP will guide towards proper conservation management of the remaining population of wild lions in Nigeria. To achieve this, DNA was extracted from all samples collected from YGR (n= 836) and KLNP (n= 93). Samples confirmed to belong to lions were further PCR amplified and genotyped for individual identification and also genetic variation at nine microsatellite loci. The number of microsatellite loci was increased to nine in this study so as to enhance our precision of individual identification.

### Microsatellite amplification and genotyping

All lion samples were PCR amplified and scored for allelic variability at nine polymorphic microsatellite loci (FCA001, FCA008, FCA026, FCA031, FCA045, FCA077, FCA126, FCA506 and FCA567; Menotti-Raymond *et al.* 1999) in paper V. PCRs were done in a GeneAmp 9700 thermocycler (Applied Biosystems). Primers were multiplexed in batches based on differences in fragment length and dye. The primer combinations were as follows: FCA001-FCA026-FCA031, FCA008-FCA045-FCA126, and FCA077-FCA506-FCA567. After amplification, alleles of the PCR products of the multiplex three loci, labelled with different dyes and of different lengths were separated using capillary electrophoresis in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

Alleles were sized relative to GS500 ROX size standard and proof read and scored in Geneious vs. 5.6.6 (Biomatters).

### Molecular sexing

Sex of identified individuals were determined using X and Y chromosome specific primers (SMCX17 and DBY7; Hellborg & Ellegren 2004). The primers have been designed to avoid non target amplification (Taberlet & Luikart 1999). After amplification, 2.5 µl of each PCR product was evaluated using 2% agarose gel with GelRed™ (Biotium) staining and samples with one band were scored as females (XX) and samples with two bands as males (XY).

### Data analysis

We used the identity analysis module in the program CERVUS (Marshall *et al.* 1998) to identify individuals with unique genotypes within the data set. We also calculated number of alleles (K), Allelic richness (A), observed ($H_{OBS}$) and expected ($H_{EXP}$) levels of heterozygosity, $P_{(ID)}$ and $P_{(ID)sibs}$ from the microsatellite genotype data. The software program CREATE (Coombs *et al.* 2008) was used to create input files for use in the software program FSTAT v2.93 (Goudet 2002). Inbreeding coefficient ($F_{IS}$), population fixation index ($F_{ST}$) and Jost's estimate of genetic differentiation ($D_{est}$) were calculated using FSTAT and GenAlEx 6.5 (Peakall & Smouse 2006). Test for deviations from Hardy-Weinberg equilibrium exact test within populations was calculated based on 1000 randomisations, bootstrapping over loci at 95% CI. The nominal statistical significance value of 5/100 was adjusted for multiple comparisons using the Bonferroni correction to minimize possible type I error. $F_{ST}$ is used instead of $R_{ST}$ (Slatkin 1995) because it is considered to be a more reliable estimate of genetic differentiation when using small data set with less than 20 loci.

# RESULTS AND DISCUSSION

### Trend of events in lion decline in West Africa

In paper I of this thesis we investigated the trend of events for the decline of lions in general, with special focus on the West African lions. We reviewed as far back as possible articles on lions and we also used anecdotal references from various sources. Various books traced the fossils of the lions back to as late as 2-1.5 million years ago in West, East and South Africa, where they spread over to Europe and America 1 million years later (Werdelin & Lewis 2005, Antunes et al. 2008, Barnett *et al.* 2009). Historically the lions were known to roam all over Africa including the Sahara desert, Europe and the Middle East. The lion population began to decline about 2000 years ago due to anthropogenic activities that led to its disappearance from most places including Europe (Antunes *et al.* 2008). Some researchers have recently made efforts using different methods to estimate the population of lions (Ferreras & Cousins, 1996; Chardonnet, 2002; Bauer & Merwe, 2004; IUCN, 2006a & b; Riggio *et al.*, 2013). Several of these studies have reported drastic declines both in population and range sizes of the lions over the years (Nowell & Jackson, 1996, Chardonnet, 2002, Bauer & Merwe, 2004, Wilson & Mittermeier, 2009, Packer *et al.* 2013), most of which are attributed largely to anthropogenic activities. With increase in anthropogenic pressure lions have now largely disappeared from unprotected areas, especially in West Africa. The most recent article by Riggio *et al.* (2013) reviewed all available data combined with satellite images and estimated that 32,000 lions are confound in 67 areas comprising of 3.4 million $km^2$ which is 17% of its historical range or about 25% of savannah Africa.

Hoffman *et al.* (2010) carried out assessment of the status of the world's vertebrates using data for 25,780 species from

the IUCN Red List of threatened species. They have shown how the status of different vertebrates has changed over time, with about 52 species of mammals moving closer to extinction each year.

### Preservation medium for lion faecal sample DNA

DNA obtained from non-invasive samples like faeces is often degraded (low in quantity and quality; Taberlet *et al.* 1999). Therefore we tested three different preservation media in paper II with the aim of getting the best medium for preserving lion faecal sample DNA prior to further analysis. Of the twenty-three samples analyzed, the cytochrome *b* gene was successfully amplified in all three preservation methods, except for one sample that was only amplified from the two-way storage medium. Evaluation of mtDNA amplification showed no apparent difference in the strengths of the bands amplified from the samples in the three different methods of preservation.

Amplification of the nuclear DNA of the 20 lion samples from the three different storage media using the six microsatellite loci showed a significantly higher amplification success for samples stored in ethanol than in ASL buffer or two-step storage (Fig. 4). Amplification success for samples stored in

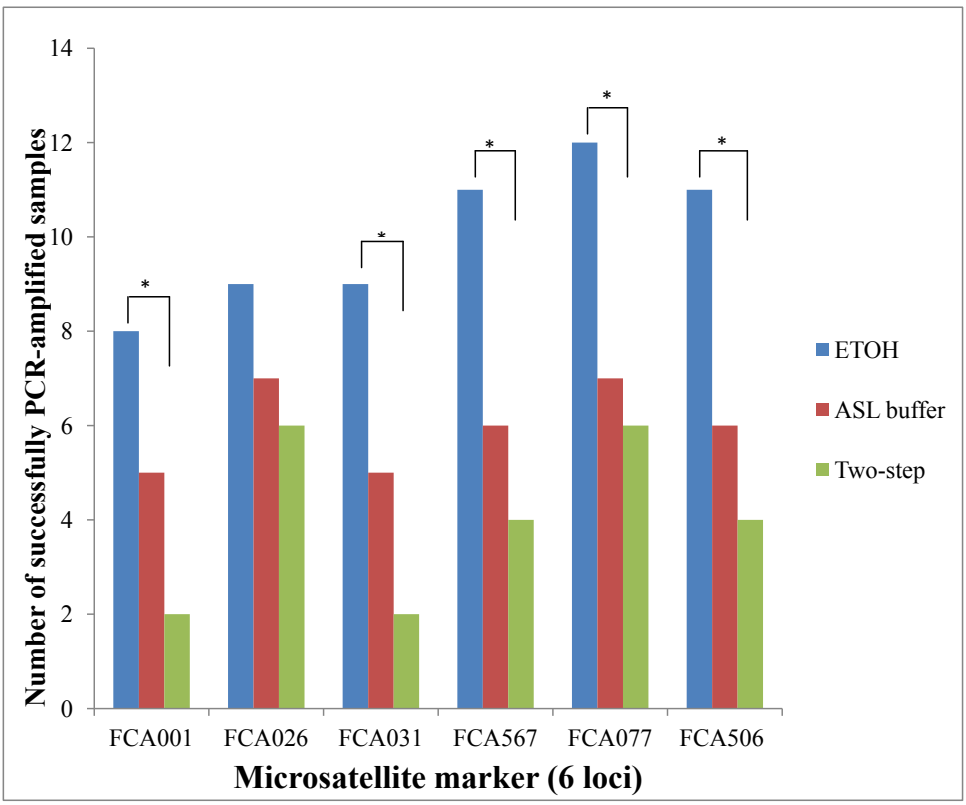


**Figure 4:** Number of successfully genotyped samples after three repeated independent PCRs from the different medium: ethanol (ETOH), ASL buffer (ASL) and Two-step storage, respectively. Stars indicate pair-wise significant ($P < 0.05$) differences (Chi-square).

ethanol was 50%, ASL buffer was 30% while two-step storage was 20% (n=20 individuals, 6 loci). DNA amplification success also differed significantly between the loci. On average, the highest amplification was obtained at locus FCA077. However, the non-significant interaction between preservative method and loci indicates that the probability of obtaining a positive outcome at any locus was not influenced by the preservation method used. Several studies have tried to evaluate the best or most reliable method for the preservation of faecal sample DNA in 70-100% ethanol, dry silica gel, DMSO, EDTA, Tris and salt (DETs), freezer or air-dried (Wasser *et al.* 1997, Frantzen *et al.*1998, Murphy *et al.* 2000, Nsubuga *et al.* 2004). The different studies show different results depending on study species and environmental conditions at study sites. In our study mitochondrial DNA was more easily amplified than the nuclear DNA. The majority of samples amplified using primers for mitochondrial DNA (only 3% PCR failure), and the bands were easily evaluated in 2% agarose gels and the sequences were of high quality. For the nuclear markers, i.e. the six microsatellite loci, direct and parallel comparison of the amplification success of the three medium of storage showed that samples preserved in ethanol amplified most successfully which makes 95% ethanol an ideal medium of storage for lion faecal DNA for genetic studies aiming for individual identification, population monitoring and population size estimates.

**Phylogenetic origins of Nigerian lions**

The aim of paper III was to determine the pattern of phylogenetic distribution of the Nigerian lions and lions in other parts of Africa and India. This will give an idea about where to focus conservation efforts in the future when the need arises for decision making. Sequences were obtained for all eight samples analyzed covering 944 bp of the cytochrome *b* gene. The Nigerian lion grouped together with high bootstrap support (96 %) with lions from West and Central Africa, including India (Fig.5). We found that lions from KLNP were more similar to lions in Benin for the mitochondrial sequence than to the population in YGR. Moreover, the YGR lion population was more similar to the Cameroon lion population. This was also depicted in the phylogenetic trees based on the sequence data where KLNP lion clustered with lions in Benin in 83% of bootstrap replication and YGR lion clustered closely with lion in Cameroon in 92% bootstrap replications.

**Feasibility of lion faecal sample DNA from tropical region**

Before embarking on a large-scale study it is important to conduct a pilot study to know the feasibility and applicability of non-invasive sampling in population study of a new species (Taberlet *et al.*1999). In paper IV of this thesis we conducted a pilot study to test the feasibility and reliability of using lion faecal sample DNA collected from tropical regions to identify individuals as well as determine genetic diversity within YGR. A total of 108 samples were extracted. We successfully amplified DNA from 43 of these samples (40%). The 33 samples confirmed to belong to lions were genotyped for individual identity and also scored for allelic variation at two microsatellite loci (Ple53 and Ple56; Gaur *et al.* 2006). Sixteen individuals were identified if assuming that all of the obtained genotypes represented unique individuals and 11 individuals if we assume that allelic drop out has affected our genotyping. The lions here were found to exhibit some inbreeding ($F_{IS}$=0.21). Both microsatellite markers showed relatively high genetic variation. Our survey is original in its application here.

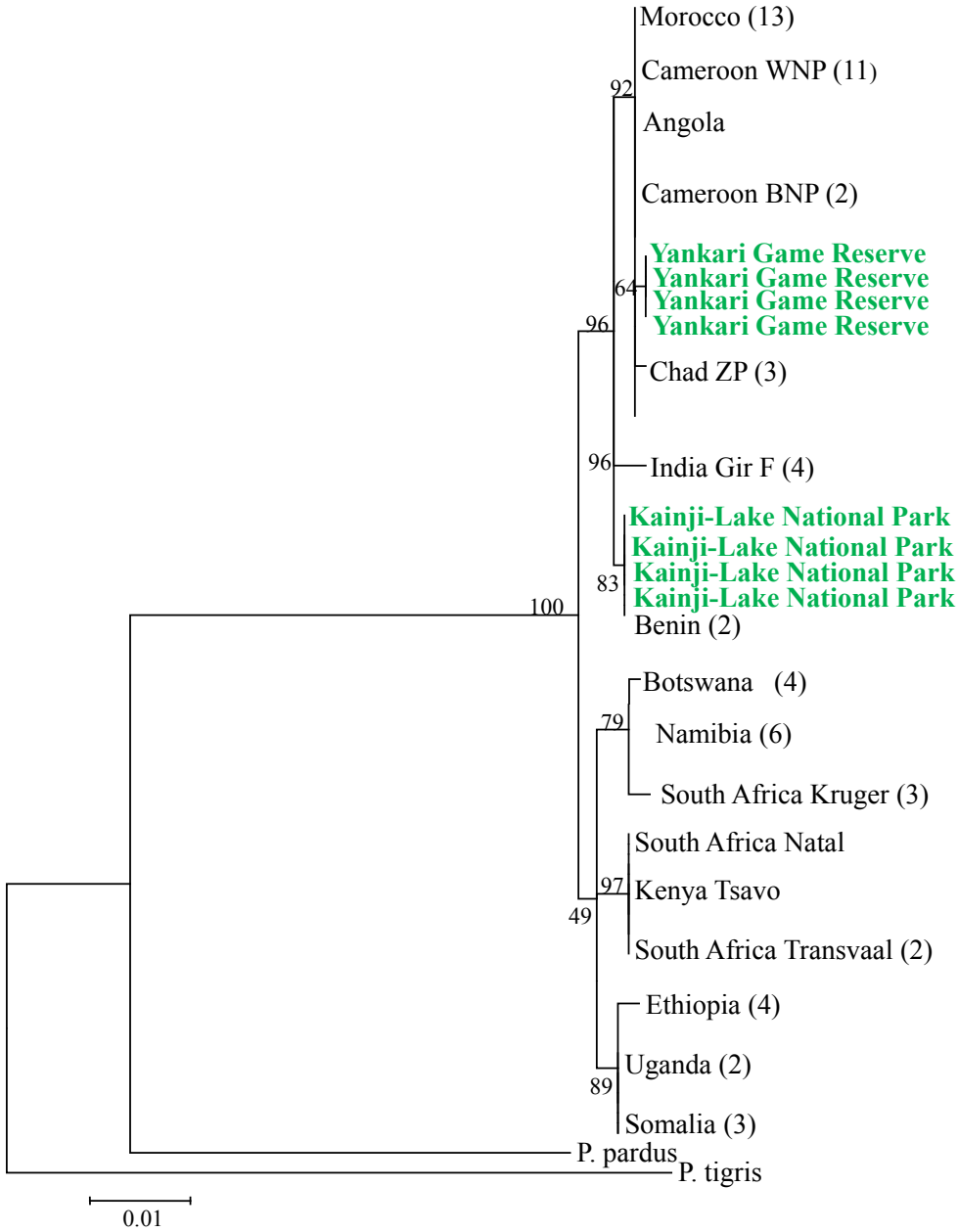


**Figure 5:** Phylogenetic tree from Maximum Likelihood analysis of lion mitochondrial cytochrome *b* sequences. Numbers in bracket represent the number of lion sequences downloaded from GenBank for each haplotype and area. Abbreviations are as follows: Cameroon BNP (Cameroon Benuoe National park), Cameroon WNP (Cameroon Waza National Park), Chad ZP (Chad Zakouma National park), South Africa Transvaal, South Africa Kruger National Park. Highlight in green are individuals from YGR & KLNP.

**Population size and level of gene flow between Nigerian wild lion populations**

In paper V we aimed at estimating the population size and genetic diversity of lions within the two protected areas in Nigeria, YGR and KLNP. Individuals were genotyped at nine polymorphic microsatellite loci where a total of eight individuals were identified in YGR if we assume that allelic drop-out had affected the scored genotypes, while ten individuals were identified in KLNP (Tables 1 & 2). In both YGR and KLNP all individuals were sampled within the core area of the study sites. The number of individuals estimated in YGR in the present study is lower than the number recorded during the pilot survey conducted by Tende *et al.* (2010) where eleven individuals were identified from two microsatellite loci. This difference could be due to either a disappearance of individuals from the population due to natural deaths or to the activities of poachers within the reserve. The population of lions in both KLNP and YGR exhibit significant signs of inbreeding which is not surprising given their small population sizes (Table 3). The inbreeding found in YGR in the present study is in line with our pilot study conducted in 2008 (Tende *et al*. 2010) when the inbreeding coefficient was estimated to be 0.21, whereas in this present survey the value was found to be 0.49. The inbreeding levels in both YGR (0.49) and KLNP (0.38) are high and comparable to what has been recorded in some other carnivore species (e.g. Scandinavian wolf; Laikre & Ryman 1991, Liberg *et al*. 2005, Bensch *et al*. 2006). For instance, the estimated inbreeding coefficient in the Scandinavian wolf was ranging up to 0.41 before the population was rescued by a single immigrant from Finland (Vila *et al*. 2003). The arrival of this immigrant into the Scandinavian wolf population provided the possibility to avoid inbreeding, decrease the risk of inbreeding depression and cause population growth. High inbreeding coefficient reaching up to 0.37 has also been recorded in the brown bear (Laikre *et al*. 1996). Some studies (e.g. Lande 1995, Laikre 1999, Bijlsma *et al*. 2000, Keller & Waller 2002) have shown that if populations remain small and isolated for many generations they are bound to face increased inbreeding and gradual erosion of genetic variability. In Nigeria the two lion populations are small, isolated and restricted to the two areas of study (YGR and KLNP). This is a threat to their long-term survival. There was no sign of any gene flow between the YGR and KLNP populations ($F_{ST}$ = 0.17, Dest = 0.65). This is not surprising due to the fact that the two populations are small and isolated from each other with a large geographical distance. The two protected areas are about 1000 km apart and separated by several dispersal barriers including highways, agricultural landscapes, human settlements and cities with no corridor for possible dispersal. This isolation is expected to build up the observed pattern of allelic differentiation between the two populations.

**Table 1**: Identified individuals in Yankari Game Reserve. Shown are; Sample identity (SampleID), individual identity (IndvID), sex and allelic length at the nine scored loci, *** (indicates missing data).

| | | | | **Microsatellite Loci** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SampleID | IndvID | Sex | #times sampled | FCA001 | FCA026 | FCA031 | FCA567 | FCA007 | FCA506 | FCA008 | FCA045 | FCA126 |
| YGR321 | Y#1 | - | 4 | 129/155 | 128/128 | ***/*** | 85/85 | 135/144 | 244/244 | ***/*** | 125/125 | 124/124 |
| YGR242 | Y#2 | F | 107 | 127/127 | 128/137 | 242/244 | 94/96 | 146/153 | 214/214 | 117/117 | 127/127 | 124/124 |
| YGR211 | Y#3 | - | 5 | 129/155 | 128/128 | ***/*** | 85/85 | 130/135 | 244/244 | ***/*** | 125/125 | 124/124 |
| YGRB1 | Y#4 | M | 6 | 127/129 | 128/130 | ***/*** | 85/85 | 135/141 | 244/244 | 124/124 | 125/125 | 124/124 |
| YGRN1 | Y#5 | M | 21 | 127/127 | 128/128 | 242/242 | 94/105 | 141/153 | 191/191 | 117/117 | 127/127 | 127/127 |
| YGR13 | Y#6 | F | 14 | 127/127 | 128/137 | 244/244 | 96/105 | 141/153 | 191/214 | 117/117 | 127/127 | 127/127 |
| YGR56 | Y#7 | F | 1 | 137/155 | 130/130 | ***/*** | 85/85 | 135/135 | ***/*** | ***/*** | 125/125 | 124/124 |
| YGR7 | Y#8 | - | 1 | 127/127 | 137/137 | 242/244 | 94/107 | 153/153 | 214/214 | 117/117 | 127/127 | ***/*** |

**Table 2:** Identified individuals in Kainji-Lake National Park. Shown are: Sample identity (SampleID, individual identity (IndvID), sex and allelic length at the nine scored loci, *** (indicates missing data)

**Microsatellite loci**

| SampleID | IndvID | Sex | #times sampled | FCA001 | FCA026 | FCA031 | FCA567 | FCA077 | FCA506 | FCA008 | FCA045 | FCA126 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KLNP13** | K#1 | M | 4 | 127/129 | 130/137 | 234/246 | 98/103 | 148/153 | 193/*** | 129/133 | 127/127 | ***/*** |
| **KLNP18** | K#2 | M | 1 | ***/*** | 122/130 | 224/224 | 78/78 | ***/*** | 229/229 | 129/129 | ***/*** | 129/191 |
| **KLNP19** | K#3 | M | 1 | ***/*** | 139/141 | 238/238 | 81/85 | 137/141 | 203/214 | 125/125 | 139/153 | 133/151 |
| **KLNP4** | K#4 | M | 3 | ***/*** | 134/139 | 238/252 | 83/85 | 137/139 | 195/203 | 119/133 | 139/139 | 139/139 |
| **KL26** | K#5 | M | 1 | 145/145 | 141/141 | 238/238 | 83/85 | 137/153 | 195/195 | 125/125 | 139/139 | 133/133 |
| **KL18** | K#6 | F | 7 | ***/*** | 137/137 | 246/246 | 103/105 | 148/151 | 191/191 | 133/133 | 145/149 | 131/139 |
| **KL11** | K#7 | F | 7 | 153/153 | 134/141 | 238/238 | 83/85 | 137/139 | 195/195 | 125/125 | 139/139 | 133/133 |
| **KL9** | K#8 | M | 1 | 129/129 | 128/130 | 234/234 | 98/111 | 148/153 | 85/191 | 133/133 | ***/*** | 139/139 |
| **KL33** | K#9 | F | 1 | 153/153 | 134/139 | ***/*** | 85/85 | 153/153 | 193/208 | 117/133 | ***/*** | 133/133 |
| **K22** | K#10 | M | 1 | 145/153 | 144/148 | 244/244 | 83/94 | 137/*** | 197/203 | 127/129 | 149/153 | 133/*** |

**Table 3:** Summary of genetic diversity; number of alleles (K), allelic richness (A), sample size (N), Observed and Expected heterozygosity ($H_{OBS}$ & $H_{EXP}$) and inbreeding coefficient ($F_{IS}$) in the two populations over the years

| | *Yankari Game Reserve (N = 8)* | | | | | | *Kainji-Lake National Park (N=10)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | N | K | A | $H_{OBS}$ | $H_{EXP}$ | $F_{IS}$ | N | K | A | $H_{OBS}$ | $H_{EXP}$ | $F_{IS}$ |
| FCA001 | 8 | 4 | 3.61 | 0.50 | 0.60 | 0.29 | 5 | 4 | 4.00 | 0.25 | 0.82 | 0.72 |
| FCA008 | 5 | 3 | 2.77 | 0.20 | 0.51 | 0.63 | 10 | 6 | 4.06 | 0.40 | 0.77 | 0.50 |
| FCA026 | 8 | 3 | 2.88 | 0.37 | 0.62 | 0.41 | 10 | 9 | 5.66 | 0.70 | 0.90 | 0.23 |
| FCA031 | 4 | 2 | 2.00 | 0.50 | 0.65 | 0.14 | 10 | 6 | 4.71 | 0.20 | 0.83 | 0.76 |
| FCA045 | 8 | 2 | 2.00 | 0.00 | 0.53 | 1.00 | 8 | 6 | 4.49 | 0.57 | 0.79 | 0.29 |
| FCA077 | 8 | 6 | 4.37 | 0.70 | 0.80 | 0.07 | 8 | 6 | 4.61 | 0.83 | 0.87 | -0.05 |
| FCA126 | 8 | 2 | 2.00 | 0.00 | 0.53 | 1.00 | 8 | 5 | 3.80 | 0.37 | 0.71 | 0.49 |
| FCA506 | 7 | 3 | 2.44 | 0.14 | 0.69 | 0.86 | 8 | 7 | 5.05 | 0.50 | 0.85 | 0.43 |
| FCA567 | 8 | 5 | 3.93 | 0.50 | 0.72 | 0.32 | 10 | 9 | 5.43 | 0.80 | 0.87 | 0.08 |
| **Mean** | - | 3.33 | 2.88 | 0.32 | 0.63 | 0.49 | - | 6.44 | 4.64 | 0.51 | 0.82 | 0.38 |

## Conclusions and Further research

This study has shown that the populations of wild lions that still exist in Nigeria are small and isolated from each other as recorded by the lack of gene flow within and between them. Small populations are susceptible to chance events. The influence of stochastic variation in demographic (reproduction and mortality) rates is usually high causing random fluctuation in size. The smaller the population size the greater the probability that fluctuations will lead to extinction. Because mating is prone to occur between individuals that are closely related in small populations, recessive deleterious alleles might be brought to limelight in homozygous form in the offspring. This increases the chances of offspring being affected by deleterious effects on all aspects of reproduction and survival, which could elevate the risk of extinction on the level of the population. Moreover, the low level of genetic variation in each of the populations indicates elevated risks of extinction due to inabilities to adapt to sudden environmental change.

If this iconic African species is going to persist in the future, then it is very important that measures be put in place to mitigate the negative effect of inbreeding (inbreeding

depression) which, though not obviously now, might be seen in the near future. It will be vital to make plans for exchange of individuals between populations with neighboring countries like Cameroon and Benin as this study has also found out that the Nigerian lions have two different origins; each originating from neighboring countries like Cameroon and Benin. The bringing together of genetically dissimilar mates, hybrid vigor can be advantageous because it will enhance reproductive success. Another source for possible increase in genetic diversity could be zoo lions; there are surprisingly many lions in zoological gardens in Nigeria (e.g. Jos zoological garden houses over 5 lions within a small cage; pers. obs.). To carry out successful transfer, there will be a need to first investigate the genetic origins of lions in these zoological gardens.

The creation of corridor for dispersal between populations is an option to connect subpopulations. This could help to reduce isolation that builds up the observed pattern of allelic differentiation between the two populations. But this does not appear realistic for the lion populations in YGR and KLNP due to the high human and livestock densities characterizing most of the surroundings of these protected areas. These can increase mortality risk of cubs and "possible dispersers" because of overlap with human habitations and livestock, which may act as barriers to gene flow between the two populations and possible populations in neighboring countries.

The existence and maintenance of genetically diverse populations with good connectivity between subpopulations are essential factors for long-term viability of a population and should be the primary target of any acceptable conservation management program.

It is important for a monitoring program to be put in place for the continuous monitoring of the demographic and genetic status of the Nigerian lion in order to gauge their realistic chances of future survival and reproduction. The rigorous and continuous monitoring of these populations will provide an ideal opportunity to follow the changes in population size and genetic diversity within and between them over time.

# REFERENCES

Afolayan, T.A. 1978. The effect of fires on the vegetation in Kainji Lake National Park, Nigeria. *Oikos* 33: 376-382.

Antunes A, Troyer, J.L., Roelke, M.E., Pecon-Slatery, J., Packer, C., Winterbach, C., Winterbach, H., Hemson, G., Frank, L., Stander, P., Siefert, L., Driciru, M., Funston, P.J., Alexander, K.A., Prager, K.C., Mills, G., Wildt, D., Bush, M., O'Brien, S.J., Johnson, W.E. 2008. The Evolutionary Dynamics of the Lion *Panthera leo* Revealed by Host and Viral Population genomics. PLoS Genetics 4(11): e1000251.

Barnett, R., Yamaguchi, N., Barnes, I., Cooper, A. 2006. The origin, current diversity, and future conservation of the modern lion(*Panthera leo*). Proceedings of the Royal Society Bulletin 273: 2159-2168.

Barnett, R., Shapiro, B., Barnes, I., Ho, S.Y.W., Burger, J., Yamaguchi, N., Higham, G., Wheeler, H.T., Rosendahl, W., Sher, A.V., Sotnikova, M., Kuznetsova, T., Baryshnikov, G., Martin, L., Harington, R., Burns, J., Cooper, A. 2009. Phylogography of lions (Panthera leo ssp.) reveals three distinct taxa and a late Pleistocene reduction in genetic diversity. *Molecular Ecology* 18: 1-10.

Beebee, T., Rowe, G. 2008. An introduction to molecular Ecology. Oxford university press

Bhagavatula, J., Singh, L. 2006. Genotyping faecal samples of Bengal tiger (Panthera tigris tigris) for population estimation: A pilot study. *BMC Genetics* 7: 48.

Bhargava, A., Fuentes, F. F. 2010. Mutational Dynamics of Microsatellites. *Molecular Biotechnology* 44: 250-266.

Balme, G. A., Hunter, L.T.B., Slotow, R. 2007. Evaluating Methods for Counting Cryptic Carnivores. *Journal of Wildlife Managemen*t

73 (3): 433- 441.

Bauer, H., Vander Merwe, S. 2004. Inventory of free ranging lions *Panthera leo* in Africa. *Oryx* 38: 26-31.

Bayes, M. K., Smith, K. L., Alberts, S. C., Altmann, J., Bruford, M.W. 2000. Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. Conservation *Genetics* 1: 173-176.

Bertola, L. D., Van Hooft, W. F., Vrieling, K., Uit de Weerd, D. R., York, D. S., Bauer, H., Prins, H. H. T., Funston, P. J., Udo de Haes, H. A., Leirs, H., VanHaeringen, W. A., Sogbohossou, E., Tumenta, P.N., De Iongh, H. H. 2011. Genetic diversity, evolutionary history and implications for conservation of the lion (*Panthera leo*) in West and Central Africa. *Journal of Biogeography* 38: 1356-1367.

Bensch, S., Andren, H., Hansson, B., Pedersen, H-C., Sand H., Sejberg, D., Wabakken, P., Åkesson, M., Liberg, O. 2006. Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS ONE* 1(1):e72. doi:10.1371/journal.pone.0000072.

Bijlsma, R., Bundgaard, J., Boerema, A. C. 2000. Does inbreeding affect the extinction risk of small populations?: predictions from Drosophila. *Journal of Evolutionary Biology* 13: 502-514.

Björklund, M. 2003. The risk of inbreeding due to habitat loss in the lion (*Panthera leo*). *Conservation Genetics* 4: 515-523.

Boulanger, J., Himmer, S., Swan, C. 2004. Monitoring of grizzly bear population trends and demography using DNA mark–recapture methods in the Owikeno Lake area of British Columbia. *Canadian Journal of Zoology* 82: 1267-1277.

Briker, J., Bushar, L. M., Reinert, H. K., Gelbert, L. 1996. Purification of high quality DNA from shed skin. *Herpetology Review* 27: 133-134.

Brown, J. L., Bush, M., Packer, C., Pusey, A. E., Monfort, S. L., O'Brien, S. J., Janssen, D. L., Wildt, D. E. 1991. Developmental changes in pituitary-gonadal function in free-ranging lions [Panthera leo leo) of the Serengeti Plains and Ngorongoro Crater. *Journal of Reproduction and Fertility* 91: 29- 40.

Callens, T., Galbusera, P., Matthysens, E., Durand, E.Y., Githiru, M., Hyughe, J.R., Lens, L. 2011. Genetic signature of population fragmentation varies with mobility in seven bird species of a fragmented Kenyan cloud forest. *Molecular Ecology* doi:10.1111/j.1365-294X.2011.05028.x.

Ceballos, G., Ehrlich, P. R. 2002. Mammalian Population Losses and the Extinction Crisis. *Science* 296(5569): 904-907.

Chardonnet, P. 2002. Conservation of the African lion: contribution to a status survey. Paris: International Foundation for the Conservation of Wildlife.

Coombs, J.A., Letcher, B.H., Nislov, K.H. 2008. CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. *Molecular Ecology Resources* 8: 578-580 .Vs.1.31.

Craigie, I. D., Baillie, J. E. M., Balmford, A., Carbone, C., Collen, B., Green, R.E., Hutton, J.M. 2010. Large Mammal population declines in Africa's protected areas. *Biological conservation* 143: 2221-2228.

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

Crick, H.Q.P., Marshall, P.J. 1981.The birds of Yankari Game Reserve, Nigeria: their abundance and seasonal occurrence. *Malimbus* 3: 103-113.

De Barba, M., Waits, L.P., Genovesi, P., Rand, E., Chirichella, R., Cetto, E. 2010. Comparing opportunistic and systematic sampling methods for noninvasive genetic monitoring of a small translocated brown bear population. *Journal of Applied Ecology* 47: 172-181.

Dunham, J., Peacock, M., Tracy, C.R., Nielsen, J., Vinyard, G. 1999. Assessing extinction risk: integrating genetic information. *ConservationEcology*3: 2. http://www.consecol.org/vol 3 / iss1/art 2/.

Ellegren, H. 2004.Microsatellites: Simple Sequences with Complex Evolution. Nature Reviews 5: 435- 445.

Erlich, H.A., Gelfand, D., Sninsky, J.J. 1991. Recent advances in the polymerase chain reaction. *Science* 252: 1643-1651.

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

Estes, J.A., Terborgh, J., Brashares, J.S., Power, M.E., Berger, J., Bond, W.J., Carpentar, S.R., Essington, T.E., Holt, R.D., Jackson, J.B.C., Marquis, R.J., Oksanen, R., Oksanen, T., Paine, R.T., Pikitch, E.K., Ripple, W.J., Sandin, S.A., Scheffer, M., Schoener, T.W., Shurin, J.B., Sinclair, A.R.E., Soule, R.V., Wardle, D.A. 2011. Trophic downgrading of planet earth. Science 333: 301-306.

Falconer, D.S., Mackay, T.F.C. 1996. Introduction to quantitative genetics. Harlow, UK Longman.

Ferreras, P., Cousins, S.H. 1996. The use of a delphi technique with GIS for estimating the global abundance of top predators: the lion in Africa. Zoology Department, Oxford University.

Frankham, R. 1995. Inbreeding and Extinction-a Threshold Effect. *Conservation Biology* 9(4): 792-799.

Frankham, R. 1996. Relationship of genetic variation to population size in wildlife. *Conservation Biology* 10: 1500-1508.

Frankham, R., Ballou, J.D., Briscoe, D.A. 2002. Introduction to Conservation Genetics. Cambridge University Press, Cambridge, UK.

Frankham, R., Kingslover, J.G. 2004. Response to environmental change: Adaptation or extinction. In: Ferrier R., Dieckman U., Couvet D (Eds.) Evolutionary Conservation Biology. Cambridge University Press, Cambridge pp 85-100.

Frantzen, M.A.J., Silk, J.B., Ferguson, J.W.H., Wayne, R.K., Kohn, M.H. 1998. Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology* 7: 1423-1428.

Gagneux, P., Boesch, C., Woodruff, D.S. 1997. Microsatellite scoring errors associated with noninvasive genotyping based on nuclear DNA amplified from shed hair. *Molecular Ecology* 6: 861- 868.

Gaur, A., Shailaja, K., Singh, A., Arunabala,V., Satyarebala, B,Singh, L. 2006. Twenty polymorphic microsatellite markers in the Asiatic lion (*Panthera leo persica*). *Conservation Genetics* 7: 1005-1008.

Geerling, C. 1973. The vegetation of Yankari Game Reserve, its utilization and condition. *Bulletin* 3: Department of Forestry, University of Ibadan.

Gerloff, U., Schlotterer, C., Rassmann, K., Rambold, I., Hohmann, G., Fruth, B., Tautz, D. 1995. Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). *Molecular Ecology* 4: 515-518.

Gilpin, M.E., Soule, M.E., 1986. Minimum viable populations: processes of species extinction. In: Soule, M.E. (Ed.), Conservation Biology: The Science of Scarcity and Diversity.

Green, A.A. 1989. Avifauna of Yankari Game Reserve, Nigeria: New records and observations. *Malimbus* 12:31-51.

Gobush, K., Kerr, B., Wasser, S. 2009. Genetic relatedness and the disrupted social structure in a poached population of African elephants. *Molecular Ecology* 18:722-734.

Goossens, B., Waits, L.P., Taberlet, P. 1998. Plucked hair samples as a source of DNA: reliability of dinucleotide microsatellite genotyping. *Molecular Ecology* 7:1237-1241.

Goudet, J. 2002. FSTAT a computer program to estimate and test gene diversities and fixation indices Version 2.9.3.2.

Hellborg, L., Ellegren, H. 2004. Low levels of nucleotide diversity in mammalian Y chromosomes. *Molecular Biology and Evolution* 21:158-163.

Hoffmann, M., Taylor, C.H., Angulo, A *et al*. 2010. The impact of Conservation on the status of the World'sVertebrates.sciencexpress. org/2/10.1126/science.1194442.

Höss, M., Kohn, M., Pääbo, S., Knauer, F., Schröder, W. 1992. Excrement analysis by PCR. *Nature* 359: 199.

Howe, J., Obbard, M.E., Schaefer, J.A. 2006. Extirpation risk of an isolated black bear population under different management scenarios. *Journal of Wildlife Management* 71(2): 603-612.

Higgins K., Lynch, M. 2001. Metapopulation extinction caused by mutation accumulation. *Proctocol of National Academic of Science USA*

98:2928-2933.

IUCN, 2004. Red List of Threatened Species. Available from:http://www.redlist.org/.

IUCN. 2006a. Conservation strategy for the lion in west and central Africa. IUCN SSC Cat Specialist Group, Gland, Switzerland and Cambridge, UK.

IUCN. 2006b. Conservation strategy for the lion in eastern and southern Africa. IUCN SSC Cat Specialist Group, Gland, Switzerland and Cambridge, UK.

Keller, L.F., Waller, D.M. 2002. Inbreeding effects in wild populations. *Trends in Ecology and Evolution* 17:230-241.

Kissui, B.M., Packer, C. 2004. Top-down regulation of a top predator: lions in the Ngorongoro Crater. Protocol of the Royal Society Series Bulletin 271: 1867-1874.

Kohn M., Wayne, R. K. 1997. Facts from feces revisited. *Trends in Ecology and Evolution* 12: 223-227.

Kohn, M.H., York, E.C., Kamradt, D.A., Haught, G., Sauvajoy, R.M., Wayne, R. K. 1999. Estimating population size by genotyping faeces. *Protocol of the Royal Society Bulletin* 266: 657- 663.

Kruckenhauser, L., Rauer, G., Däubl, B., Haring, E. 2009. Genetic monitoring of a founder population of brown bears (*Ursus arctos*) in central Austria. *Conservation Genetics* 10: 1223-1233.

Lacy, R.C. 2000. Considering threats to the viability of small populations using individual-based models. *Ecological Bulletins* 48: 39-52.

Laikre, L, Ryman, N. 1991. Inbreeding depression in a captive Wolf (*Canis lupus*) population. *Conservation Biology* 5: 33- 40.

Laikre, L., Andren, R., Lars, H., Ryman, N. 1996. Inbreeding depression in Brown Bear (*Ursus arctos*). *Biological Conservation* 76: 69-72.

Laikre, L. 1999. Conservation genetics of Nordic carnivores: lessons from zoos. *Hereditas* 130: 203-216.

Lande, R. 1988. Genetics and demography in biological conservation. *Science* 214: 1455-1460.

Lande, R. 1993. Risks of population extinction from demographic and environmental stochasticity and random catastrophes. *The American society of Naturalists* 142: 911-927.

Lande, R. 1995. Mutation and conservation. *Conservation Biology* 9:7 82-791.

Lewonti, R.C. 1991. Twenty-five years ago in Genetics: Electrophoresis in the development of evolutionary Genetics: Milestone or Millstones?. *Genetics* 128: 657-662.

Liberg, O., Andren, H., Pedersen, C.H., Sand, H., Sejberg, D., Wabakken, P., Åkesson, M., Bensch, S. 2005. Severe inbreeding depression in a wolf *Cani lupus* population. *Biology Letters* 1(1): 17-20.

Litt, M., Luty, J.A. 1989. A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. American Journal of Human Genetics 44 (3): 397- 401.

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

McBride, C. 1977. The white lions of Timbavati. E. Stanton.

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

McKelvey, K.S., Schwartz, M.K. 2004. Genetic errors associated with population estimation using non-invasive molecular tagging: problems and new solutions. *Journal of Wildlife Management* 68: 439- 448.

Menotti-Raymond, M., David, V.A., Lyons, L.A., Schaffer, A.A., Tomlin, J.F., Hutton, M.k., O'Brien, S.J. 1999. A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics* 57: 9-23.

Miller, C.R., Waits, L.P. 2003. The history of effective population size and genetic diversity in the Yellowstone grizzly (*Ursus arctos*): Implications for conservation. *Protocol of the National Academic of Sciences USA* 100: 4334-4339.

Mills, L.S., Citta, J.J., Lair, K., Schwartz, M., Tallmon, D. 2000. Estimating animal abundance using non-invasive DNA sampling: promise and pitfalls. *Ecological Applications* 10: 283-294.

Mondol, S., Karanth, K.U., Kumar, N.S.,

Gopalaswamy, A. M., Andheria, A., Ramakrishnan, U. 2009. Evaluation of non-invasive genetic sampling methods for estimating tiger population size. *Biological Conservation* 142: 2350-2360.

Morin, P.A., Woodruff, D.S. 1996. Noninvasive genotyping for vertebrate conservation. In: Molecular Genetic Approaches in conservation (Smith TB & Wayne RK). pp. 298-313. Oxford University Press, Oxford.

Mullis, K.B., Faloona, F.A. 1987. Specific synthesis of DNA *in ritro* via a polymerase catalyzed chain reaction. *Methods in Enzymology* 155: 335-350.

Mullis, K. 1990. The unusual origin of the polymerase chain reaction. *Scientific American* 262(4): 56-65.

Munso, L., Brown, J.L., Bush, M., Packer, C., Jansen, D., Reiziss, S.M., Wildt, D. E. 1996. Genetic diversity affects testicular morphology in free-ranging lions (*Panthera leo*) of the Serengeti Plains and Ngorongoro Crater. *Journal of Reproduction and Fertility* 108:11-15.

Murphy, R., Sites, J.W.J., Buth, D., Haufler, C. 1996. Proteins: isozyme electrophoresis. In:. Hillis D,Moritz C, Mable B, eds. Molecular Systematics Sunderland, MA: Sinauer Associates, Inc, pp 51-120.

Murphy, M.A., Waits, L.P., Kendall, K.C. 2000. Quantitative evaluation of faecal drying methods for brown bear DNA analysis. *Wildlife Society Bulletin* 28: 951-957.

Nsubuga, A.M., Robbins, M.M., Roeder, A.D., Morin, A., Boesch, C., Vigilant, L. 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular Ecology* 13: 2089-2094.

Nowell, K., Jackson, P. (Compilers and editors) .1996. Wild cats: Status survey and Conservation Action Plan. IUCN/ SSC Cats Specialist Group. IUCN, Gland, Switzerland.

O'Brien, S.J., Roelke, M.E., Marker, L., Newman, H., Winkler, C.W., Meltzer, D., Colly, L., Evermann, J., Bush, M., Wildt, D.E. 1985. Genetic basis for species vulnerability in the cheetah. *Science* 227: 1428-1434.

Packer, C., Pusey, A.E., Gilbert, D.A., Martenson, J., O'Brien, S.J. 1991. Case study of a population bottleneck: lions of the Ngoronogoro Crater. *Conservation Biology* 5: 219-230.

Packer, C., Loveridge, A.,Canney, S., Caro, T., et al. 2013. Conserving large carnivores: dollars and fence. *Ecology letters* Doi:10.1111/ele.12091.

Paetkau, D. 2003. An empirical exploration of data quality in DNA-based population inventories. Molecular Ecology 12: 1375-1387.

Panasci, M., Ballard, W.B., Breck, S., Rodriguez, D., Densmore III, L.D., Wseter, D.B., Baker, R.J. 2011. Evaluation of faecal DNA preservation techniques and effects of sample age and diet on genotyping success. *Journal of Wildlife Management*. 75(7): 1616-1624.

Peakall, R., Smouse, P.E. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288-295.

Perez, I., Geffen, E., Mokady, O. 2006. Critically Endangered Arabian leopards *Panthera pardus nimr* in Israel: estimating population parameters using molecular scatology. *Oryx* 40: 295-301.

Piggott, M.P., Taylor, A.C. 2003. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. *Australian Journal of Zoology* 51: 341-355.

Poole, K.G., Mowat, G., Fear, D.A. 2001. DNA-based population estimate for grizzly bears *Ursus arctos* in northeastern British Columbia, Canada. *Wildlife Biology* 7: 105-115.

Pokhriyal, B., Thorat, K., Limaye, D.A., Joshi, Y.M., Kadam, V. J., Dubey, R. 2012. Microsatellite markers – a novel tool in Molecular Genetics. *International Journal of Research in Pharmacy and Chemistry* 2 (2): 2231- 2781.

Pompanon, F., Bonin, A., Taberlet, P. 2005. Genotyping errors: causes, consequences and solutions. *Nature* 6: 847-859.

Proctor, M.F., McLellan, B.N., Strobeck, C., Barclay, R.M.R. 2005. Genetic analysis reveals demographic fragmentation of grizzly bears yielding vulnerably small populations. *Protocol of the Royal Society Bulletin* 272: 2409-2416.

Riggio, J., Jacobson, A., Dollar, L., Bauer, H., Becker, M., Dickman, A., Funston, P., Groom, R., Henschel, P., De Iongh, H., Lichtenfeld, L., Pimm, S. 2012. The size of savannah Africa: A lion's (*Panthera leo*) view. Biodiversity Conservation 22: 17-35.

Rudnick, J.A., Katzner, T.E., Bragin, E.S., Rhodes, E.O. Jr., Dewoody, J.A. 2005. Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. *Molecular Ecology* 14: 2959-2967.

Rutledge, L.Y., Holloway, J.J., Patterson, B.R., White, B.N. 2008. An improved field method to obtain DNA for the identification from wolf scat. *Journal of Wildlife Management* 73 (8): 1430-1435.

Saccheri, I., Kuussaari, M., Kankare, M., Vikman, P., Fortelius, W., Hanski, I. 1998. Inbreeding and extinction in a butterfly metapopulation. *Nature* 392: 49- 494.

Saiki, R.K., Scharf, S., Faloona, F.A., Mullis, K.B., Horn, C.T., Erlich, H.A., Amheim, N. 1985. Enzymatic amplification of Beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350-1354.

Saiki, R., Gelfand, D., Scharf, S., Higuchi, R., Horn, G., Mullis, K., Erlich, H. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487- 491.

Scaglione, F.E., Schröder, C., Degiorgi, G., Zeira, O., Bollo, E. 2010. Cranial Malformations in Related White Lions (*Panthera leo krugeri*). *Veterinary Pathology* DOI: 10.1177/0300985810382518.

Schwartz, M.K., Luikart, G., Waples, R.S. 2007. Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution* 22: 25-33.

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139: 457- 462.

Spong, G., Johansson, M., Bjorklund, M. 2000. High genetic variation in leopards indicates large and long-term stable effective population size. *Molecular Ecology* 9: 1773-1782.

Sunnucks, P. 2000. Efficient genetic markers for population biology. *Trends in Ecology and Evolution* 15 (5): 199-203.

Taberlet, P., Griffin, S., Goossens, B. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research* 24 :3189-3194.

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

Taberlet, P., Luikart, G. 1999. Non-invasive genetic sampling and individual identification. *Biological Journal of the Linnean Society* 68: 41-55.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* 17: 6463-6471.

Tende, T., Ottosson, U., Hansson, B., Åkesson, M., Bensch, S. 2010. Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling. *African Journal of Ecology* 59: 7-12.

Trinkel, M. et al. 2008. Translocating lions into an inbred lion population in the Hluhluwe-iMfolozi Park, South Africa. *Animal Conservation* 11:138-143.

Trinkel, M., Cooper, D., Packer, C., Slotow, R. 2011. Inbreeding depression increases susceptibility to bovine tuberculosis in lions: An experimental test using and inbred-outbred contrast through translocation. *Journal of Wildlife Diseases* 43: 494-500.

Turner, I.M. 1996. Species loss in fragments of tropical rain forest: area view of the evidence. *Journal of Applied Ecology* 33: 200-209.

Vallet, D., Petit, E., Gatti, S., Levréro, F., Ménard, N. 2007. A new 2CTAB/PCI method improves DNA amplification success from faeces of Mediterranean (Barbary macaques) and tropical (lowland gorillas) Primates. *Conservation Genetics* DOI 10.1007/s10592-007-9361-8.

Valsecchi, E., Glockner-Gerrari, D., Ferrari, M., Amos, W. 1998. Molecular analysis of the efficiency of sloughed skin sampling in whale population genetics. *Molecular Ecology* **7**:

1419-1422.

Vila, C., Sundqvist, A.K., Flagstad, O. 2003. Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. *Protocol of the Royal Society Bulletin* 270: 91-97.

Vynne, C., Baker, M.R., Breuer, Z.K., Wasser, S.K. 2011. Factors influencing degradation of DNA and hormones in maned wolf scat. *Animal Conservation* Doi:10.1111/j.1469-1795.2011.00503.x.

Wasser, S.K., Houston, C.S., Koehiler, G.M., Cadd, G.G., Fain, S.R. 1997. Techniques for applications of faecal DNA methods to field studies of Ursids. *Molecular Ecology* 6: 1091-1097.

Waits, L. P., Luikart, G., Taberlet, P. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Molecular Ecology 10: 249-256.

Waits, L.P., Paetkau, D. 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. *Journal of wildlife management* 69 (4): 1419-1433.

Waits, L.P., Beason-Buckley, V.A., Johnson, W.E., Onorato, D., McCarthy, T. 2006. A select panel of polymorphic microsatellite loci for individual identification of snow leopards (*Panthera uncia*). *Molecular Ecology* 7:311-314.

Wandeler, P., Smith, S., Morin, P.A., Pettifor, R.A., Funk, S.M. 2003. Patterns of nuclear DNA degeneration over time - a case study in historic teeth samples. *Molecular Ecology* 12: 1087-1093.

Werdelin, L. & Lewis, M.E. (2005) Plio-Pleistocene Carnivora of eastern Africa: species richness and turnover patterns. *Zool J Linn Soc* 144: 121-144.

Wilcox, B. A., Murphy, D. D. 1985. Conservation Strategy: The Effects of Fragmentation on Extinction. *The American Naturalist* 125 (6): 879-887.

Wildt, D.E., Bush, M., Goodrowe, K.L., Packer, C., Pusey, A.E., Brown, J.L., Joslin, P., O'Brien, S.J. 1987. Reproductive and genetic consequences of founding isolated lion populations. *Nature* 329: 328-331.

Wilson, D.E., Mittermeier, R.A. (chief Eds). 2009. Handbook of the Mammals of the World, vol. 1, Carnivores, LynxEdicions (in association with Conservation International and IUCN), Barcelona.

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

Wright, P.A., Wynford-Thomas, D. 1990. The polymerase chain reaction: miracle or mirage? A critical review of its uses and limitations in diagnosis and research. *Journal of Pathology* 162: 99-117.

# POPULATION SIZE AND GENETIC DIVERSITY OF NIGERIAN LIONS *(PANTHERA LEO)*

## POPULAR SUMMARY

Lions (*Panthera leo*) have become known to be synonymous with wild Africa since its extinction from most parts of its former range (Europe, Asia and Southern America). Few people realize that anthropogenic activities have caused habitat loss, fragmentation and reduction in size to the lions, forcing them to the brink of extinction. Whenever out in nature, most tourists that visit national parks or game reserves spend most of their time searching for, or observing and admiring lions they have encountered. The lion, Africa's most iconic species, has attracted so much admiration to itself as some countries or clubs have incorporated its picture on their logo or coat of arms (e.g. lion's club international, United Kingdom of Britain, Northern Ireland, Estonia, and Kenya) to mention a few. Yet this prestigious species is in dire danger of extinction in near future if measures are not put in place. Currently the lion is being listed as "vulnerable" on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species. It is now classified as "Endangered" in West and Central Africa where their relict populations still exist. They have vanished from over 80% of their historic range, and currently they exist in only 28 countries in Africa and at one locality in India. And even within Africa only seven countries (Botswana, Ethiopia, Kenya, South Africa, Tanzania, Zambia and Zimbabwe) are believed to still have more than 1,000 lions in the wild. This alarming decline calls for a need to gather information on population size and level of gene flow that may exist within and between populations within the different countries that still harbor lions. This is vital in order to devise conservation and management measures for the long-term survival of the few populations left. This project was born out of a desire to know the number of lions that exist within Yankari Game Reserve (YGR) when I was employed there by the Leventis foundation in 2006 as a research officer. I got involved in discussion with some staff of the reserve to have an idea of the number of lions that exist there. All conversation was met with many different "guess estimates" of between 50-100 individual lions. And yet within a reserve with such a small landmass (2,244km$^2$) there were too few encounters of lions when out in the field, if such number is assumed to exist. Thus we designed a survey to estimate the population size of the lions in YGR using direct count. During the period of one year of the survey, there were very few encounters of lions to make available analyzable data; but there was often encounter of lion foot-prints and faeces when we go out in the field for survey. Thus we resolved to the use of lion faecal collection after Ulf Ottosson consulted with Staffan Bensch and other members of the Molecular Ecology and Evolution Lab at the Department of Biology (Bengt Hansson

and Mikael Åkesson). Through the review of articles and books we investigated the trend of events for the decline of lions. This was to establish the lion's historical range as far back as possible by describing the decrease in the lion population with references to human population, anthropogenic effects and climatic events etc. By so doing we can understand the trend of events and thereby assist on devising means to overcome these factors to save the remaining population. Lion faeces were collected opportunistically along existing game viewing and patrol tracks within the Reserve and preserved in 95% ethanol prior to analysis in the laboratory. Although the use of faeces for population and genetic study appears promising, it has a number of pitfalls associated with it, because of degradation of DNA (low quantity and quality) in faeces, which can cause genotyping errors. Therefore we deemed it important to store faecal samples in conditions that can preserve the minute DNA quantity at the time of collection before laboratory analysis. We tested three preservatives and found that ethanol was better among the other preservatives used. We investigated the pattern of distribution and extent of overlap of lions in Nigeria and other parts of Africa and India in order to understand the genetic makeup of the Nigerian lion within the West and Central African range. This we did by analyzing lion sequence data obtained from Genbank with sequences from eight supposedly unrelated individual lions already identified from both YGR and KLNP. An investigation into the genetic makeup and phylogeographic history of the lions in Africa is important for understanding both the evolutionary processes affecting them as well as developing conservation strategies and thus make future management decisions easier. A pilot study was conducted within YGR to test the feasibility and reliability of obtaining quality DNA from faecal sample collected from a tropical environment to identify individuals. This method proved feasible and reliable, and eleven individuals were identified using two polymorphic microsatellite loci. The success of the pilot study prompted us to extend the study to the second protected area within Nigeria, Kainji-Lake National Park (KLNP) that still holds lions. The aim was to estimate the population size of lions within these two areas to gather information about the number of wild lions that still exist in Nigeria, and also to understand the level of gene flow that may exist between them. We found that about eight lions still exist in YGR, while ten individuals were estimated in KLNP. The two populations were found to exhibit signs of inbreeding with no sign of gene flow between them. The finding in this study is an important guide for the conservation of lions in Nigeria as well as those in the neighboring countries of Cameroon, Benin and Burkina Faso.

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# TACK!

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# POPULATIONS AND GEOGRAPHICAL DISTRIBUTION OF LIONS *(PANTHERA LEO)* IN WEST AFRICA FROM ANCIENT TO PRESENT TIMES, ESPECIALLY DURING THE LAST 200 YEARS

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# POPULATIONS AND GEOGRAPHICAL DISTRIBUTION OF LIONS *(PANTHERA LEO)* IN WEST AFRICA FROM ANCIENT TO PRESENT TIMES, ESPECIALLY DURING THE LAST 200 YEARS

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## ABSTRACT

The lion can be traced back until about 2 million years ago (MYA) and historically it was found all over Africa, in Europe and parts of Asia, and together with its close relatives also in to the Americas. In this survey, we aim to establish the lion's historical range and population size as far back as possible. By describing the decrease in the lion population with references to human population, anthropogenic effects and climatic events etc. we want to shed light on the trends responsible for the population's decline, and assist on devising means to overcome these factors to save the remaining population.

**Keywords:** lion, Panthera leo, lion population, history of the lion

# INTRODUCTION

Lion (*Panthera leo*) fossils can be traced back to the Late Pliocene in Western Africa and Early Pleistocene in Eastern and Southern Africa, coinciding with the flourishing of grasslands there some 2-1.5 million years ago (MYA) (Werdelin & Lewis, 2005, Antunes et al., 2008). 1 MY later, lions had spread to Europe. Between about 130,000 – 10,000 years ago, during the Late Pleistocene, lions extended their range from Africa not only into large parts of Eurasia but also into the Americas, having the largest intercontinental distribution for any large land mammal, except man (Kurtén, 1968; Barnett et al., 2006, 2009).

Historically, this top-predator lived virtually all over Africa, including in what is now the Sahara desert (fig 1; table 1). It was also widespread in southern Europe and eastwards through the Middle East to the Indian subcontinent. Maybe more than a million lions roamed the Earth at that time. But, around 2,000 years ago the lion populations had already begun to decline, basically for two reasons - the expansion of human populations and climate change.

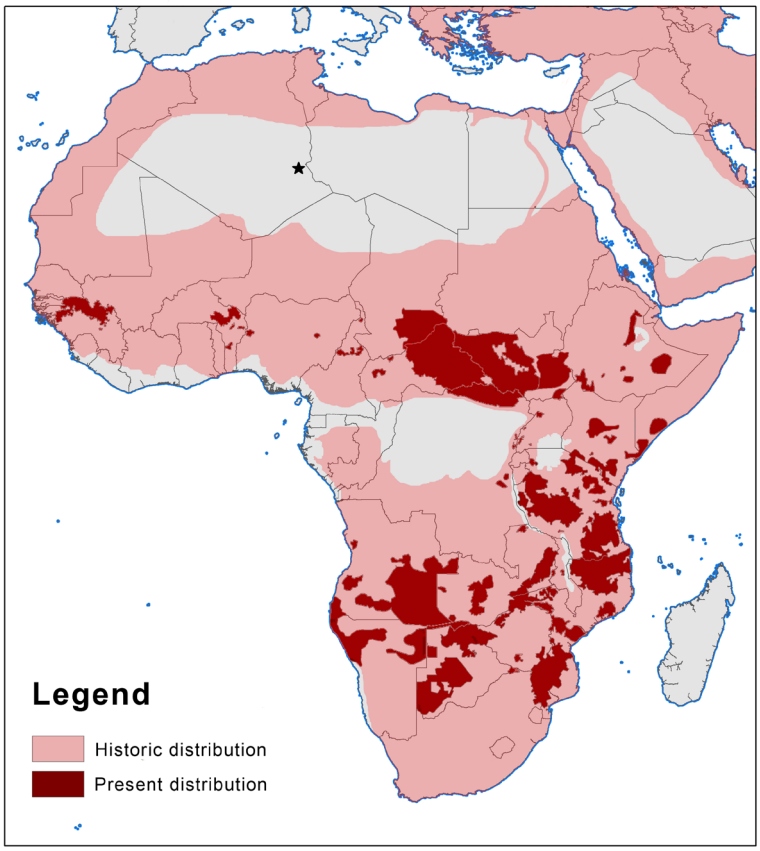


**Figure 1.** Historical and current distribution of lions in Africa adopted after Reggio et al. (2013) and Panthera (www.panthera.com). The black star show the position of the rock paintings at the Iherir Plateau, Algeria (Plate 1).

This led to its disappearance from its last stronghold in Europe and Greece during the first century AD (e.g. Bauer & Van der Merwe, 2002, 2004; Antunes et al., 2008; Wilson & Mittermeier, 2009).

In parts of Europe, the lion (cave lion, a subspecies of modern day lion) that man painted on the walls in France and elsewhere around 15 000 yrs ago, died out before historic times (Kurtén, 1968). Lions survived longer in remote parts of Greece. For instance when Xerxes marched through Greece 480 BC his baggage camels were attacked by lions and Herodotus also considered them common at that time. By 300 BC Aristotle found them rare and by 100 AD they were gone, no doubt due to competition from the numerically increasing human populations and more sophisticated developments. They survived in what was then the Holy Land, into the Crusades and well into the last century in Syria, Iran and Iraq (from Schaller, 1972). This decline progressed gradually through the Middle East and beyond, so that today the only remnant lion population outside Africa is the one in the Gir Forest of north western India, counting perhaps 400 animals (of the subspecies *persica*; Wildlife Trust of India, 2011).

Although the lions disappeared from the expanding Saharan desert areas in central West Africa, they still survived in good numbers both north and south of the Sahara. To the north, e.g. in the present day Algerian Atlas mountains, they were quite common as late as the middle 1800s. A French colonial officer, Lt. Jules Gerard of the 3rd Spahi-regiment, wrote a famous and well-illustrated book about lion-hunting this late in lion history this far north in Africa (Gerard, 1855). But soon the lions would get extinct in northern Africa too, in Tunisia 1891 and in Algeria 1893. The last "Barbary lion" of northern Africa was shot in 1920, although they may in fact have survived in the High Atlas Mountains until the 1940s (Nowell & Jackson, 1996).

Since the 1940s, when the number of lions in Africa was estimated to be 450,000 (National Geographical Society, 2013), the populations have blinked out across the whole continent. Today lions are declining drastically both in population and range size across Africa. Several surveys have reported

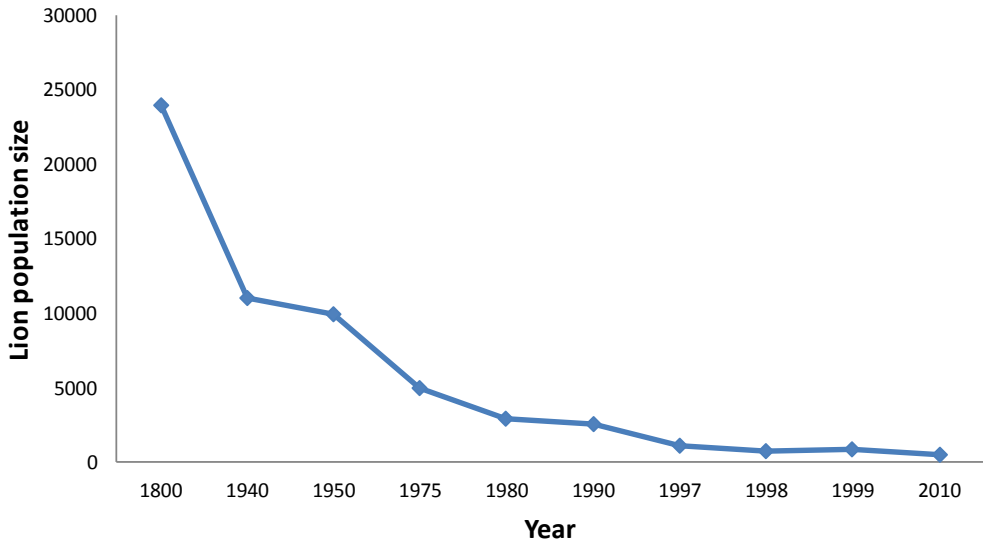


**Figure 2**. Population size of the African lion in West Africa from 1800 to present.

**Table 1.** Historical and more recent population estimates for the whole of Africa and West Africa respectively.

| | | Population estimates | | | |
|---|---|---|---|---|---|
| **References** | **year** | **Africa, total** | **Range** | **West Africa** | **Range** |
| National Geographic | 1800 | 1200000 | - | 24000[1] | - |
| National Geographic | 1940 | 450000 | - | 11000[2] | - |
| Myers 1975 | 1950 | 400000 | - | 10000[2] | - |
| Myers 1975 | 1975 | 200000 | - | 5000[2] | - |
| Ferreras & Cousins 1996 | 1980 | 76000 | - | 3000[2] | - |
| Nowell & Jackson (1996) | 1990 | 65000 | 30000-100000 | 2600[2] | 1200-400 |
| Chardonnet (2002) | 2002 | 39000 | 29000-47000 | 1163 | 968-1358 |
| Bauer et al. (2003) | 2003 | - | - | 800 | 600-1100 |
| Bauer & Van der Merwe (2004) | 2004 | 23000 | 16500-30000 | 850 | 450-1300 |
| Reggio et al. (2013) | 2010 | 34907 | - | 480 | - |

[1]With an estimated 2% of the world population 1.2 M
[2]With 4% of the African Population (c.f. Chardonnet, 2002, Bauer & Van der Merwe, 2004)

this drastic decline over the years (Nowell & Jackson, 1996; Chardonnet, 2002; Bauer & Van der Merwe, 2004; Wilson & Mittermeier, 2009; Packer et al., 2013). Now they may be as few as a total of 20,000 in the whole of Africa and a mere 500-1000 in West Africa (table 1).

In West Africa, the lion was quite widespread until rather recently. When its distribution there was at its peak it covered all of Western Africa from the Bay of Guinea to the Mediterranean Sea. Some thousand years ago, when there was much more rainfall than today, the present Sahara desert was a rich savannah type of landscape with lakes and running rivers (Gasse, 2000; Kuper & Kröpelin, 2006; Kröpelin et al., 2008) - full of game animals! This, to some extent, we know from a combination of paleontological and archaeological findings as well as from the fantastic rock-paintings found in many areas of the central Saharan mountains. For instance, in 1933 an officer of the colonial French Camel Corps, Lt. Brenans, on a reconnaissance ride along Wadi Djerat in the Tassili-n-Ajjer Mountains in southern Algeria, encountered some marvellous rock-paintings showing a profusion of elephants (*Loxodonta africana*), rhinos (probably black rhino; *Ceratotherium simum*), giraffes (*Giraffa camelopardalis*) and even hippopotamuses (*Hippopotamus amphibious*). They indicate that when these paintings were made, this present day super dry desert area was something totally different. These rock-paintings have since been found in many parts of the central Saharan mountains. They were particularly intensely studied in the 1950s, by French scientists under the leadership of Henri Lhote (e.g. Lhote, 1958). They showed that lions were quite common there about 5,000-3,000 years ago, that is before the drier climate leading to the present desert began to take its biological toll. There are, for example, some famous paintings of both lions making attacks, and of humans hunting lions dating from those days, in the Iherir Mountains (good colour reproductions in Burenhult, 1982, see Plate 1).

As to the West African areas south of the Sahara, we know there were many lions in most places even outside protected areas

**Plate 1.** Rock-painting from the Enrakle site, Iherir Plateau, Tassili n'Ajjer, Algeria. The scene shows a lion chasing a group of men with wigs and body paint. Ca 3500 BC. Foto: Göran Burenhult 1981.

until rather recently. When Mungo Park did his classic trips from Gambia towards the Niger River in the late 1700s and early 1800s (Park, 1799) he encountered lions in several places. And, much later, between 1920-1930s when the Swedish engineer and explorer Mike Joslin travelled in the "Gold Coast" areas (present day Ghana and Upper Volta; e.g. Joslin, 1947, 1950), lions and leopards (*P. pardus*) were regarded as common pests there. Other game-animals, today gone from these areas (e. g. elephant, black rhinoceros, African buffalo (*Syncerus caffer*), hartebeests (*Alcelaphus buselaphus*) and even gorilla (*Gorilla gorilla*) were quite common!

Today, with the human population in these areas having quadrupled since about 1960, and still increasing at an exponential rate, there is no more room for predators like the lion, or its natural prey outside protected areas. Thus, the total West African population of lions is now down to around 500 individuals - and the decrease is still ongoing (e.g. Riggio et al., 2013).

Before the beginning of the present century only a few efforts had been made to estimate the numbers of lions and their historical change in Africa (e.g. Myers, 1975, 1986; Ferreras & Cousins, 1996). Myers (1975) wrote, "Since 1950, lion numbers may well have been cut in half, perhaps to as low as 200,000 or even less in all ". Later, Myers (1986) wrote, "In light of evidence from all the main countries of its range, the lion has been undergoing decline in both range and numbers, often an accelerating decline, during the past two decades". In the early 1990s, IUCN SSC Cat Specialist Group members made educated "guesstimates" of 30,000 to 100,000 for the African lion population (Nowell & Jackson, 1996).

To estimate the lion population is

an ambitious exercise involving many uncertainties. Five relatively recent efforts (Ferreras & Cousins, 1996; Chardonnet, 2002; Bauer & Van Der Merwe, 2004; IUCN, 2006a & b; Riggio et al., 2013) all use different methods. The African Lion Working Group compiled individual population estimates primarily from protected areas (23,000 lions: Bauer & Van Der Merwe, 2004). For the year 1980, Ferreras & Cousins (1996), from a GIS model, predicted 18,600 lions to occur in protected areas in the whole of Africa. This will give approximately 3,000 lions in West Africa. This was probably an underestimate, as not all protected areas inhabited by lions at that time were included. Still, even if the comparison suggests that the number of lions in African protected areas has remained stable or possibly increased over time, they have declined in West Africa. Further, Ferreras and Cousins (1996) predicted that most lions in 1980 were found outside protected areas; but if this could be true for Africa as a whole it was probably not true for West Africa. Chardonnet (2002) found that unprotected areas still comprise a significant portion (c. half) of the lion's current African range. Comparison of Ferreras and Cousin's (1996) prediction of a total African lion population of 75,800 lions in 1980 (roughly three lion generations ago) to Chardonnet's (2002) estimate of 39,000 lions yields a suspected decline of 48.5%. This calculation suggests a substantial decline in lions outside protected areas over the past two decades. However, Ferreras and Cousins (1996) may have over-estimated the African lion population in 1980, as their number was derived from a model rather than from actual lion counts. So it is possible that the rate of decline of the African lion population may be lower. A group exercise led by WCS and the IUCN SSC Cat Specialist Group estimated that 42% of major lion populations were declining (IUCN, 2006a & b; Bauer, 2008). The rate of decline is most unlikely to have been as high as 90%, as reported in a series of news reports in 2003 (Kirby, 2003; Frank & Packer, 2003).

A recent article (Riggio et al., 2013) reviewed all available data combined with satellite images and estimated that 32,000 lions are now found in 67 areas comprising 3.4 million km$^2$, which is 17% of its historical range or about 25% of savannah Africa.

It has been reported that West African lions are genetically different from lions in East and Southern Africa and resemble more closely Asiatic lions (Bertola et al., 2011). A recent survey has shown that lions in Yankari Game Reserve (YGR), in North East Nigeria and Kainji-Lake National Park (KLNP) Western Nigeria are few in numbers and also genetically different from each other. In mitochondrial DNA, YGR lions are more similar to lions from Cameroon and the Central African Republic and KLNP lions are more similar to lions in Niger, Benin, Burkina Faso and Guinea (Tende et al., in prep.)

Wildlife densities in the West African ecosystem have probably always been lower than in eastern Africa. This in turn implies that lion densities here should have been lower than in other areas of sub-Saharan Africa, probably around 1-20 lions/100 km$^2$ (Bauer et al., 2003) as compared to 5-55 lions/100 km$^2$ in areas like Kruger and Serengeti National parks (e.g. Shaller, 1972; Sunquist & Sunquist, 2002). With the increase of anthropogenic pressure lions have now largely disappeared from unprotected areas, especially in West Africa. Lion densities in most protected areas were around the turn of the century less than 5 lions/100 km$^2$.

## DISCUSSION

It is likely that the West African population of lions was at its highest about 6,000-5,000 BP when the vegetation in Sahara reached its maximum and at the time when, or just

before, humans started to get more settled. Since then, changed climate and increasing human population with its attendant pressure on natural resources have lead to a decline in the lion population and already there are local extinctions in many places.

Hominids and humans have one way or the other always had a link to the lion. In the early hominid history our ancestors on the savannas of eastern and southern Africa were potential prey to the lions, leopards and other predators. Later the lion became part of the mythical world and a symbol for strength, but also a competitor for prey. The lion has been, and somehow still is, sought after for trophy hunting, though now fortunately more often hunted by camera.

Today, even though there still are occasions where people get killed by lions, man has turned the table around and now kills much more lions than he becomes a victim of. Yet it seems that the history of human-lion conflicts may have started as far back as when man began rearing cattle and other ruminating mammals and lions started to use them as part of their diet. In West Africa, Cattle grazing by nomadic herdsmen probably played a significant role in reinforcing the human-lion conflict. This is because grazing within protected areas potentially reduces food available for some of the natural preys of lions (e.g. antelopes), and consequently increasing the incidences of lions preying on cattle and likewise the persecution of lions by aggrieved herdsmen. Efforts must be directed at reducing this human-lion conflict through enhanced animal husbandry practices that reduce contact between man, his livestock and lions.

For long term survival, big predators like lions need relatively large blocks of continuous habitat and sufficient access to wild prey. With the increasing pace of human population growth, industrialization and agricultural developments over the last century, both habitat and prey for lions have declined widely.

The present situation in West Africa suggests that there are just two lion areas that are strongholds. These are the Arly-Singou ecosystem in Burkina Faso, and the Benoue complex in Cameroon (Bauer et al., 2008; Henschel et al., 2012; Riggio et al., 2013). Therefore, the protection of lions in West Africa has reached a very critical stage. Efforts need to be put in place to safeguard these two still viable populations. The other West African lion populations are small and geographically scattered around far apart and have very little chance to be self-sustained and survive in the long-run. To get these small remnant populations to survive, translocations from the more viable populations are probably needed. Potentially, lions in captivity could be a source for resurrecting populations in the wild, but the genetic origin and quality of those are doubtful or unknown and the practical problems with releasing former captive animals to the wild are possibly large (c.f. Hunter et al., 2013).

The West and Central African lions are genetically distinct from lions from other parts of Africa (Bertola et al., 2011) and this seems to have become like an obituary for the West African lion. However, fortunately there are still individuals out there to save and to make thrive for many generations although this may only be possible if proper management strategies are put in place. But to do this we need concerted efforts from many different stakeholders at different levels.

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# REFERENCES


ANTUNES, A, TROYER, J.L., ROELKE, M.E., PECON-SLATERY, J., PACKER, C., WINTERBACH, C., WINTERBACH, H., HEMSON, G., FRANK, L., STANDER, P., SIEFERT, L., DRICIRU, M., FUNSTON, P.J., ALEXANDER, K.A., PRAGER, K.C., MILLS, G., WILDT, D., BUSH, M., O'BRIEN, S.J. & JOHNSON, W.E. (2008) The Evolutionary Dynamics of the Lion *Panthera leo* Revealed by Host and Viral Population genomics. *PLoS Genetics,* 4(11), e1000251.

BARNETT, R., YAMAGUCHI, N., BARNES, I. COOPER, A. (2006) The origin, current diversity and future conservatiopn of the modern leion (Pamthera leo). *Proceedings of the Royal Society Series B,* 273, 2119-2125.

BARNETT R, SHAPIRO B, BARNES I, HO SYW, BURGER J, YAMAGUCHI N, HIGHAM G, WHEELER HT, ROSENDAHL W, SHER AV, SOTNIKOVA M, KUZNETSOVA T, BARYSHNIKOV G, MARTIN L, HARINGTON R, BURNS J & COOPER A. (2009) Phylogography of lions (*Panthera leo ssp*.) reveals three distinct taxa and a late Pleistocene reduction in genetic diversity. *Molecular Ecology*, 18, 1-10.

BAUER, H. & VAN DER MERWE, S. (2002) The African Lion Database. The African Lion Woking Group.

BAUER, H. DE IONGH, H.H., PRINCÉE, F.P.G. & NGANTOU, D. (2003) Research needs for lion conservation in West Africa. C. R. *Biologies*, 326, S112-S118.

BAUER, H. & VAN DER MERWE, S. (2004) Inventory of free-ranging lions *Panthera leo* in Africa. *Oryx,* 38, 26-31.

BAUER, H. (2008) Synthesis of threats, distribution and status of the lion from the two lion conservation strategies. In: B. Croes, R. Buij, H. de Iongh and H. Bauer (eds), *Management and conservation of large carnivores in west and central Africa*, pp. 13-28. Institute of Environmental Sciences (CML), Leiden University, Leiden.

BAUER, H., NOWELL, K. & PACKER, C. (2008) *"Panthera leo". IUCN Red List of Threatened Species. Version 2010.2.* International Union for Conservation of Nature.

BERTOLA, L.D., VAN HOOFT, W.F., VRIELING, K, UIT DE WEERD, D.R., YORK, D.S., BAUER, H., PRINS, H.H.T, FUNSTON, P.J., UDO DE HAES, H.A., LEIRS, H., VAN HAERINGEN, W.A., SOGBOHOSSOU, E., TUMENTA, P.N. AND DE IONGH, H.H. (2011) Genetic diversity, evolutionary history and implications for conservation of the lion (*Panthera leo*) in West and Central Africa. *Journal of Biogeography,* 38, 1356-1367.

BURENHULT, G. (1982) *Iherir – klippmålningar från stenåldern i Centralsahara*. Stureförlaget, Stockolm.

CHARDONNET, P. (2002) Conservation of the African Lion: Contribution to a Status Survey. International Foundation for the Conservation of Wildlife, France & Conservation Force, USA, Paris, France.

FERRERAS, P. & COUSINS, S.H. (1996) The use of a delphi technique with GIS for estimating the global abundance of top predators: the lion in Africa. Zoology Department, Oxford University.

FRANK, L. & PACKER, C. (2003) Letter to the editor. *New Scientist,* October 23.

GASSE, F. (2000) Hydrological changes in the African tropics since the Last Glacial Maximum. *Quarternary Science Reviews,* 19, 189-211.

GERARD, J. (1855) *Chasse au lion*. Librairie nouvelle, Paris

HENSCHEL, P., KIKI, M., SEWADE, C. & TEHOU, A. (2012) Improving the status of lions and cheetahs in their last stronghold in West Africa: the W-Arly-Pendjari Complex. Report, 1-31. NewYork, Panthera.

HUNTER, L.T.B., WHITE, P., HENSCHEL, P., BURTON, L.F.C., LOVERIDGE, A., BALME, G., BREITENMOSER, C. & BREITENMOSER, U. (2013) Walking with lions: why there is no role for captive-origin lions Panthera leo in species restoration. *Oryx*, 47, 19-24.

IUCN (2006a) *Conservation strategy for the lion in west and central Africa*. IUCN SSC Cat Specialist Group, Gland, Switzerland and Cambridge, UK.

IUCN (2006b) *Conservation strategy for the lion in eastern and southern Africa*. IUCN SSC Cat Specialist Group, Gland, Switzerland and Cambridge, UK.

JOSLIN, M. (1947) *Med Mike Joslin i obesegrade djungler*. Medéns Förlag. Stockholm.

JOSLIN, M. (1950) *Guld, pärlor och reptiler*. Forum. Stockholm.

KIRBY, A. (2003) *Africa's shocking lion loss*. http://news.bbc.co.uk/1/hi/sci/tech/3171380.stm [accessed 27 June 2013].

KUPER, R. & KRÖPELIN, S. (2006) Climate-controlled Holocene occupation in the Sahara: motor of Africa's Evolution. *Science,* 313, 803-807.

KRÖPELIN, S., VERSCHUREN, D., LÉZINE, A.M., EGGERMONT, H., COCQUYT, C., FRANCUS, P., CAZET, J.P., FAGOT, M., RUMES, B., RUSSELL, J.M., DARIUS, F., CONLEY, D.J., SCHUSTER, M., VON SUCHODOLETZ, H. & ENGSTROM, D.R. (2008) Climate-driven ecosystem succession in the Sahara: the past 6000 years. *Science,* 320, 765-768.

KURTÉN, B. (1968) *Pleistocene mammals of Europe*. Weidenfeld and Nicholson, London.

LHOTE, H. (1958) *A la découverte des fresces du Tassili*. Arthaud. Paris.

MYERS, N. (1975) The Silent Savannahs. *International Wildlife,* 5, 5.

MYERS, N. (1986) Conservation of Africa's cats: Problems and opportunities. In: S.D. Miller and D.D. Everett (eds), *Cats of the world: Biology, conservation and management*, pp. 437-446. National Wildlife Federation, Washington DC, USA.

NATIONAL GEOGRAPHIC SOCIETY. (2013) National Geographic Big Cat Initiative. http://animals.nationalgeographic.com/animals/big-cats/lion-decline-map/ [accessesd 19 August 2013].

NOWELL, K. & JACKSON, P. (1996) *Wild Cats. Status Survey and Conservation Action Plan.* IUCN, 382 pp.

PARK, M. (1954) *Travels into the interior of Africa (1799-1806)*. New Edition by J.M. Dent, London. 1954. and paperback by Eland Books, London 1983.

RIGGIO, J., JACOBSON, A., DOLLAR, L., BAUER, H., BECKER, M., DICKMAN, A., FUNSTON, P., GROOM, R., HENSCHEL, P., DE IONG, H., LICHTENFELD, L. & PIMM, S. (2013) The size of savannah Africa: a lion's (*Panthera leo*) view. *Biodiversity Conversvation,* 22, 17-35.

SHALLER, G.B. (1972) *The Serengeti Lion: A study of Predator-Prey Relations*. The University of Chigao Press, Chicago and London.

SUNQUIST, M., & SUNQUIST, F. (2002) *Wild Cats of the World*. Chicago: University of Chicago Press.

WERDELIN, L. & LEWIS, M.E. (2005) Plio-Pleistocene Carnivora of eastern Africa: species richness and turnover patterns. *Zoological Journal of the Linnean Society,* 144, 121-144.

WILDLIFE CONSERVATION TRUST OF INDIA. (2011) *Asiatic Lion – Population*. Asiatic Lion Information Centre. http://www.asiaticlion.org/population-gir-forests.htm [accessed 27 June 2013].

WILSON, D.E. & MITTERMEIER, R.A. (chief Eds) (2009) *Handbook of the Mammals of the World, vol. 1, Carnivores*. Lynx Edicions (in association with Conservation International and IUCN), Barcelona.

# EVALUATING PRESERVATION MEDIUM FOR THE STORAGE OF DNA IN THE AFRICAN LION *(PANTHERA LEO)* FAECAL SAMPLES

II

# EVALUATING PRESERVATION MEDIUM FOR THE STORAGE OF DNA IN THE AFRICAN LION *(PANTHERA LEO)* FAECAL SAMPLES

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## ABSTRACT

Lion faecal samples, collected in the field between 1 hour to 1 week after defecation were preserved in three different media (ethanol, ASL buffer and Two-step storage). The aim was to determine which faecal DNA field preservation method that best enhances PCR amplification success. Samples stored in ethanol showed a significantly higher amplification success of microsatellite loci than samples stored in the other two media. In contrast, amplification success of a mitochondrial locus was similar for the samples stored in the three media. We reviewed twelve previous studies that employed different media for the storage of faeces and, although there is not a fully consistent pattern in success levels of the different media, ethanol storage was scored highest in the majority of these tests.

**Keywords** Amplification success, Faecal sample DNA, Lion (*Panthera leo*), PCR, Preservation Medium.

# INTRODUCTION

For small and elusive wildlife populations, non-invasive DNA sampling through sources like faecal samples is a feasible method for obtaining genetic data (Kohn and Wayne 1997). Several studies have employed this method to estimate and monitor wildlife (Kohn et al. 1999, Ernest et al. 2000, Banks et al. 2002, Flagstad et al. 2004, Bensch et al. 2006, Perez et al. 2006, Zhang et al. 2009). Non-invasive sampling is suitable especially for carnivores such as lions (*Panthera leo*) that defecate along trails to mark territory boundaries (Macdonald 1980) where faecal samples are easily encountered and recovered. However, studies that employ non-invasive sampling are confronted with pitfalls like low quantity and quality DNA, which can result in low genotyping success and frequent genotyping errors (Taberlet et al. 1997).

The amount and quality of DNA initially present in faeces samples at the time of collection may change during the period of storage and will determine the amount and quality of DNA that can be extracted and used in downstream analyses. It is therefore important to transfer samples into a storage condition that best preserves the DNA prior to the laboratory analysis. The choice of a good preservation medium should be of prime importance for every genetic study, especially when collected samples cannot be processed immediately at the collection site. The identification of effective preservation methods can enhance PCR amplification success rate, increase genotyping accuracy and also increase the feasibility of using faecal DNA for genetic studies.

Faecal DNA has so far mainly been used in studies of mammals, and less often for studies of birds (Idaghdour et al. 2003, Regnaut et al. 2006). In mammals, DNA has been recovered from faeces that were preserved dried (Höss et al. 1992, Kohn et al. 1995, Foran et al. 1997, Taberlet et al. 1997), in 70% ethanol (Höss 1992, Kohn et al. 1995), in 99-100% ethanol (Gerloff et al. 1995) and frozen at -20°C (Reed et al. 1997).

Several studies have evaluated the relative genotyping success from two or more preservation media (Wasser et al. 1997, Frantzen et al. 1998, Murphy et al. 2002, Panasci et al. 2011). These studies span an array of mammal species (appendix 1) including several species of carnivores (e.g. Wasser et al. 1997, Murphy et al. 2002, Santini et al. 2007, Panasci et al. 2011, Reddy et al. 2012). A few studies comparing storage methods in tropical environments have been conducted mostly on primates (Gerloff et al. 1995, Frantzen et al. 1998, Bayes et al. 2000, Nsubuga et al. 2004 Vallet et al. 2007). Frantzen et al. (1998) attempted to evaluate the success of preserving baboon (*Papio cynocephalus urainus*) DNA in four different media and pointed out that the optimal methods can possibly vary according to species and conditions at the study sites.

In this study, we intend to compare the effectiveness of three preservation media, ethanol, ASL buffer and two-step storage (i.e. ethanol and silica gel), for the storage and preservation of lion faecal DNA obtained in Yankari Game Reserve, North-East Nigeria. The aim is to determine the preservation method that best enhances PCR amplification success of both the mitochondrial and nuclear DNA. This study is part of an ongoing research project that uses lion faecal sample DNA to monitor lion populations in protected areas in Nigeria (Tende et al. 2010).

We hypothesise that the PCR amplification success will differ between the preservatives used. The outcome of our test was evaluated in relation to a literature compilation of other studies testing preservation media.

# 1 MATERIAL AND METHODS

## 1.1 Study site

The study was conducted in Yankari Game Reserve (The reserve), central North-East Nigeria (9° 50'N and 10° 30'E), with a landmass of 2,244 km$^2$. The Reserve lies in the Sudan Savannah zone and the vegetation is composed mainly of dry savannah woodland with a narrow floodplain, bordered by patches of gallery forest and riparian forest (Geerling 1973, Crick and Marshall 1981, Green 1989). Temperature ranges between 18-24°C during the wet / harmattan season and 30-45°C during the dry hot season.

## 1.2 Faecal sampling

We conducted daily faeces surveys between January and April 2011 within the reserve by visiting tracks both in the mornings and evenings. We collected 23 faecal samples of similar age, that could be determined to be between 1hr and 1 week old because we often travelled these tracks more than once per day and at least on a weekly basis. Efforts were made to collect samples that were as fresh as possible (c.f. Piggott, 2004, Murphy et al. 2002). The freshness of faeces was determined based on moisture content, appearance and strength of odour (Vynne et al. 2011). Each of the twenty-three samples was preserved in the three different media (n=69); 95% ethanol, ASL buffer (Qiagen), and Two-step storage (ethanol and silica beads; e.g. Wasser et al. 1997, Reddy *et al*. 2012). Wherever a sample was encountered a small portion from the outer part of the faeces (c.f. Bidlack et al. 2007, Ferrando et al. 2008, Stenglein et al. 2010) was collected using dry sticks and straight away put into ethanol and ASL buffer. Each stick was discarded after each sample collection to avoid contamination. For the samples preserved with the Two-step storage method, these were first collected into 95% ethanol and kept for 24 hours at ambient temperature. Afterwards the ethanol was carefully poured off and the dry solid faecal sample was transferred into the tube containing silica beads (c.f. Roeder *et al*. 2004, Reddy *et al*. 2012). The top of the tube containing the dry silica gel and faecal sample was then carefully stuffed with cotton wool to make it air tight. All collected samples were stored at room temperature, thereafter transported to the laboratory and stored at -33°C prior to DNA extraction.

DNA extraction was carried out in a separate room exclusively dedicated to faeces and hair extractions. DNA from faeces was extracted using the stool DNA extraction protocols in QIAamp® (Qiagen) according to the manufacturer's instructions. All DNA extractions and PCRs contained a negative control (reagents only) to monitor for contamination at any step along the way.

## 1.3 Amplification of mitochondrial DNA

In order to evaluate the amplification success of the mitochondrial DNA, all extracted samples were PCR amplified using primers LIHYF (5'-ATGACCAACATTCGAAAATCWC-3') and LIHYR (5'-ATGTGGGTSACTGATGAG-3'). These primers are designed to amplify a short portion of the mitochondrial *cytochrome b* gene (206 bp) and enhance the detection of the target species but avoid the amplification of human and ungulate DNA in general (Tende et al. 2010). All amplifications were carried out using 2X Qiagen multiplex PCR kit in 10 µl reaction volume containing 5 µl Qiagen multiplex PCR buffer mix; 0.2 µM forward primers (Applied Biosystems), 0.2 µM reverse primer, 2.6 µl of water and 2 µl of DNA extract with a hot start at 95°C for 15 minutes. PCR profile consisted of 35 cycles as follows: 90°C for 30 seconds; annealing temperature of 52°C for 30 seconds with elongation period of 72°C for 30 seconds. A

blank control (reagents only) from extracted DNA process was included in all PCRs to monitor for contamination. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining.

All samples that were successfully amplified were sequenced using the forward primer (BigDye sequencing kit; Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems) for species identification. This is needed in order to make sure that the faeces used in the study are from the lion and not from other carnivores like the spotted hyena (*Crocuta crocuta*) and striped hyena (*Hyena hyena*) present in the reserve. Sequences were aligned against reference sequences of lion, spotted hyena and striped hyena obtained from GenBank. All samples assessed to belong to the lion were selected for further analysis.

### 1.4 Amplification of nuclear DNA

To evaluate amplification success and genotyping accuracy of the nuclear DNA all positive samples found to originate from lion were PCR amplified using six polymorphic microsatellite primers (FCA001, FCA026, FCA031, FCA077, FCA506 and FCA567; Menotti-Raymond et al. 1999). PCR amplifications were performed in 6 µl multiplex reactions containing 0.12 µl (concentration: 10 µM) dye-labelled (6-Fam or Hex) F-primer, 0.12 µl unlabelled R-primer (concentration: 10 µM), 3 µl of 2X Qiagen Master mix, 0.76 µl double distilled water and 2 µl DNA extract. PCRs were done in a GeneAmp 9700 thermocycler (Applied Biosystems) as follows: 95°C for 15 min; 40 cycles at 94°C 30 s, 52°C for 90 s, and 72°C 90 s; followed by an elongation period at 72°C for 10 min. Primers were multiplexed together in two batches based on differences in fragment length and dye. The primer combinations were as follows: FCA001-FCA026-FCA031, FCA567-FCA077-FCA506. Each sample and locus was PCR amplified three times in order to ascertain the results. Alleles of the PCR products were separated using capillary electrophoresis in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Alleles were sized relative to GS500 ROX size standard and proof read and scored in Gene Mapper 4.0 (Applied Biosystems). No allele was accepted unless it was detected at least twice in the three independent PCRs.

### 1.5 Data analysis

Direct and parallel comparison of the amplification success of the samples obtained from the three different media was made from the genotype result obtained from the six microsatellites, by direct count of the number of successful amplifications. Success rate was then estimated as the number of samples that amplified at a locus from each medium. We tested if the probability of success or failure (1, 0) at the six different loci is dependent on preservation method by building a generalized linear mixed model (GLMM) with a binomial error structure with the probability of success as the response variable, preservation method (Ethanol, ASL buffer or Two-step storage) and loci (1-6) as explanatory variables and sample replicates as a random factor, thus:

Success (1/ 0) = preservation method (Ethanol, ASL buffer, Two-step storage) + loci (1-6) + replicates (random factor)

The model also included two-way interactions of the explanatory variables and using stepwise backward elimination process, the final model which best explains the variability in the data was selected based on the value of the Akaike Information Criterion (AIC). The best-fit model was the one with the lowest AIC value.

## 2 RESULTS

Of the twenty-three samples collected, the *cytochrome b* gene was successfully amplified in all three preservation methods, except for one sample that was only amplified from the two-way storage medium. There was no apparent difference in the strengths of the bands amplified from the samples in the three different methods of preservation. The sequencing confirmed that in 20 of the samples the amplified *cytochrome b* gene matched to lion (after aligning our sequences to reference sequences of the lion, spotted and striped hyenas obtained from the GenBank) whereas three samples were found to belong to spotted hyena.

The overall microsatellite amplification success was 50% for samples stored in ethanol, 30% for ASL buffer and 20% for Two-step (n=20 individuals, 6 loci). Ethanol storage showed the highest amplification success at all six individual microsatellite loci, and this effect was significant when compared to Two-step storage for five of the six loci (Fig. 1). A logistic regression (Table 1) confirmed the main effect of storage method ($P < 0.001$) and also showed a significant difference between the loci ($P < 0.001$). On average, the highest amplification success was obtained at locus FCA077. The non-significant interaction between preservative method and loci (Table 1) indicates that the probability of obtaining a positive outcome at any locus was not influenced by the preservation method used.

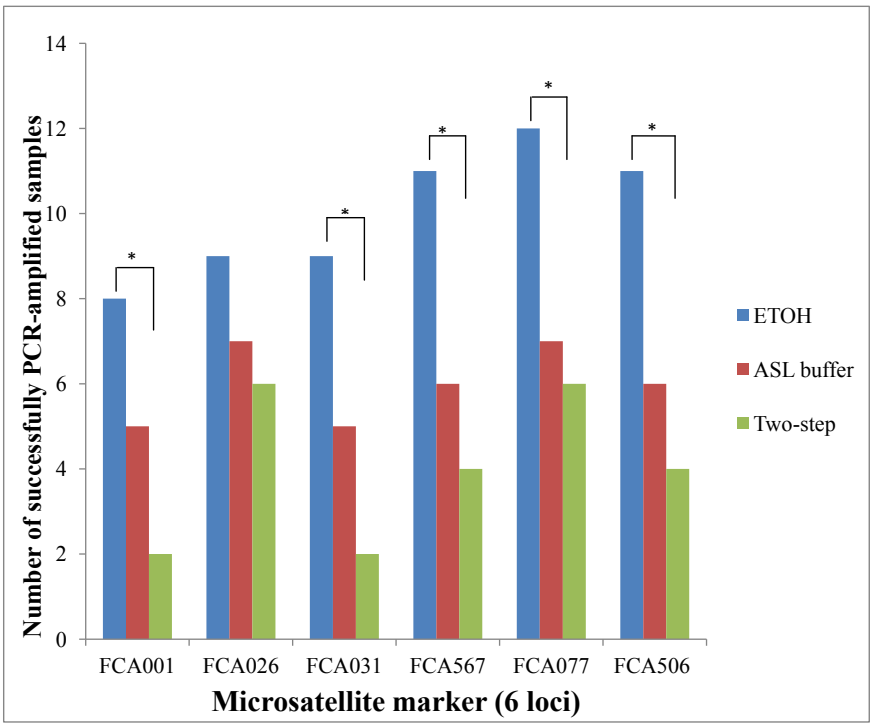


**Figure 1:** Number of successfully genotyped samples after three repeated independent PCRs from the different medium: ethanol (ETOH), ASL buffer (ASL) and Two-step storage, respectively. Stars indicate pair-wise significant ($P < 0.05$) differences (Chi-square).

**Table 1**: Logistic regression showing probability of amplification success of lion samples (n = 20) preserved in three different media.

| | Wald chi-squared | df | P |
|---|---|---|---|
| Intercept | 80.462 | 1 | <0.001 |
| Preservation method | 41.663 | 2 | <0.001 |
| Locus | 27.878 | 5 | <0.001 |
| Preservation method*locus | 11.385 | 10 | 0.328 |
| Replicates | 1.087 | 6 | 0.982 |

# 4 DISCUSSIONS

Several studies have evaluated methods for preserving DNA in faecal samples from mammals (Table 2). Although most studies compared only some of the many different media commonly used, storage in ethanol scored high in the majority of studies where it was tested. This pattern agrees with our study of lions. We found that mtDNA was amplified with a high success (97%) irrespective of the media. However for nuclear markers (the microsatellites) that are the most valuable markers for studies of individual identification and population comparisons we found the highest success for samples preserved in ethanol. This is suggesting that ethanol (>90%) is an ideal medium for preserving DNA in faecal samples from lions in studies aiming for individual identification, population monitoring and population size estimates. Our findings are thus consistent with Murphy et al. (2002) who found that silica gel beads produced poorer results compared to ethanol, and therefore recommended the use of absolute ethanol for the preservation of faecal sample DNA. They also found that brown bear (*Ursus arctos*) samples preserved in DETs buffer performed equally well as samples preserved in high percentage ethanol. Moreover, this study also reported that oven-dried samples produced better results than samples stored in silica beads or preserved by microwave drying. In the present study of lions in Nigeria, the climate is very hot (up to +45°C) and dry during most of the times faeces are found. The faeces therefore tend to dry quickly (<24 hrs) thus making it unnecessary to dry the samples before storage.

Panasci et al. (2011) showed that coyote faeces preserved in 95% ethanol and DETs buffer performed equally well and both performed better than samples stored in lysis buffer. Wasser et al. (1997) and Panasci et al. (2011) advised against the use of lysis buffer for the preservation of faecal DNA because it could result in the digestion of ingested hair from consumed prey that thereby could act as a potential for DNA contamination. Also, it is possible that faeces collected in ASL buffer get lysed if stored at room temperature prior to deposition in the freezer. This might have affected DNA quality in our case, since the collected faeces were not deposited immediately in the freezer. Other preservation methods might however also be useful for faecal DNA storage depending on storage conditions. For example, Wasser et al. (1997) found silica gel to perform well especially when stored at room temperature.

We reviewed twelve different studies that had evaluated the effects of storage medium for DNA in faecal samples based on amplification success both on mtDNA and nDNA (Appendix 1). Although these studies have not evaluated the same methods a striking pattern is that storage in ethanol (70-95%) proved to be the best medium in seven out of the twelve studies reviewed (Table 2). The two-step storage was found to be the best in three out of four studies (Table 2). Two buffers (GUS lysis buffer and RNA*later)* worked well in the few studies that tested them, and this needs to be investigated further.

**Table 2:** Summary of overview; the figures 1-3 denotes the within study results for preservation, where 1=best, 2= second best, 3= third best

| Authors (year) | Climate | Preservation methods | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Ethanol | 2-step | Buffers | | | | Silica | Drying | Freezing |
| | | | | ASL | DETs | RNA*later* | GUS | | | |
| This study | Tropical, dry | 1 | 3 | 2 | - | - | - | - | - | - |
| Reddy *et al.* 2012 | Temperate, dry | 2 | 1 | - | - | - | | 3 | - | - |
| Roeder *et al.* 2004 | Temperate | 1 | 1 | | - | - | - | 2 | - | - |
| Calderon *et al.* 2009 | Tropical, humid | 2 | - | - | - | 1 | - | 3 | - | - |
| Santini *et al.* 2007 | Temperate, dry | 2 | - | - | - | - | 1 | - | 3 | 3 |
| Frantz *et al.* 2003 | Temperate | 1 | - | - | 2 | - | - | - | - | 3 |
| Piggot & Taylor 2003 | Temperate | 1 | - | - | 2 | - | - | - | 1 | 1 |
| Murphy *et al.* (2002) | Temperate | 1 | - | - | - | - | - | 2 | 3 | - |
| Frantzen *et al.* 1998 | Temperate – Sub-tropical | 1 | - | - | 1 | - | - | - | - | 2 |
| Wasser *et al.* 1997 | Temperate | 2 | - | - | - | - | - | 1 | - | 3 |
| Nsubuga *et al.* 2004 | Tropical, humid | - | 1 | - | 2 | 2 | - | 2 | - | - |
| Panasci et al. 2011 | Temperate, dry | 1 | - | 2 | | - | - | - | - | - |

Although some studies have evaluated the relative success of the same preservation media (Table 2) they have reached different conclusions on their performance. This suggests that effectiveness varies among taxa, environmental conditions and possibly other variables. These conflicting findings call for more studies to be conducted in order to establish the medium that is best for the preservation of faecal samples. In particular, there are relatively few studies from dry tropical regions. Because most tests have been using fresh faeces (i.e. less than 12 hrs old) we still know relatively little on the storage conditions that are best for the most commonly encountered faeces in the field, i.e. those defecated days to weeks before collection.

Reddy et al. (2012) found that the amount of amplifiable DNA in faeces declined with time. This is probably because environmental conditions (e.g. sun exposure, temperature, humidity) facilitate the degradation of DNA in faeces after deposition (Morin and Woodruff 1996, Farrell et al. 2000, Goossens et al. 2000, Lucchini et al. 2002, Friedberg 2003, Nsubuga et al. 2004). The degradation of DNA in faeces appears to be faster during warm compared to cool seasons (Lucchini et al. 2002, Vynne et al. 2011). Wasser *et al.* (1997) and Vynne et al. (2011) found that this rate of DNA degradation is dependent on both the ambient temperature and humidity. It is therefore vital to recover faeces as quickly as possible (Piggott 2004, Murphy et al. 2007). Faeces freshness can be determined based on moisture content, appearance and strength of odour (Rutledge et al. 2008, Vynne et al. 2011). Vynne et al. (2011) carried out a study in the Brazilian Cerrado, a seasonally dry tropical environment to determine factors that cause DNA degradation in faeces of maned wolf (*Chrysocyon brachyurus*). They found environmental condition as one of the predictors of the amplification success of mtDNA. Thus, the time interval between scat deposition and collection, as well as sample storage duration can influence the amplification success apart from preservation method (Wasser et al.1997, Frantzen et al.1998, Goossens et al. 2000). Although efforts were made during our survey period to recover faeces as soon as possible to minimize exposure to environmental degradation, the samples were not analyzed immediately. Thus it is possible that the duration of storage of the samples in the freezer before analysis in our case (>1 month) could impact our results. This needs to be investigated further in the same species by analyzing samples of different storage periods.

Previous studies have demonstrated that the specific part of the faeces collected may affect the DNA quality and the amplification success (Rutledge et al 2008, Gobush et al. 2009, Stenglein et al. 2010, Vynne et al. 2011, Wasser et al. 2011). This is because DNA from faeces is obtained from sloughed epithelial cells of the intestinal lining of the focal species (Albaugh et al. 1992). These cells are often not homogenously distributed in the faeces (Wasser et al. 1997). The different techniques used to address the problem of non-uniform distribution of DNA are; 1) swabbing the surface of the faeces (Lampa et al. 2008, Rutledge et al. 2008, Vynne et al. 2011), 2) scraping the surface (Kohn et al. 1999, Fernando et al. 2000, Livia et al. 2007), 3) wash the surface (Banks et al. 2002; Maudet et al. 2004, Piggott 2004, Bhagavatula & Singh 2006, Perez et al. 2006), 4) sampling from the outside (Ferrando et al. 2008, Gobush et al. 2009) or 5) homogenizing the faeces before taking a sample (Wasser et al. 1997; Frantzen et al. 1998; Puechmaille et al. 2007). We collected our samples from the outer layer of each faeces. The rational of this approach is that the surface of the faeces is the last to have been in contact with the intestinal lining (Frenando et al. 2003). Samples from the surface of the faeces will thus be more likely to contain the sloughed intestinal cells

and yield good DNA quality (Reed et al. 2004, Prugh et al. 2005).

In conclusion, our results are in agreement with most studies (e.g. Santini *et al.* 2007, Calderon *et al.* 2009, Reddy *et al.* 2012) that ethanol is the best method of preservation of DNA. However, it is necessary to point out certain pitfalls associated with the use of ethanol. For example, tubes may leak during transportation when the caps are not properly tightened which may erode/destroy labelling. Also, there may be restriction on transportation by air because ethanol is highly inflammable. Therefore, and because the amplification success in relation to preservation method might vary depending on study species and condition at the study sites as mentioned previously (e.g. Frantzen et al.1998, Vynne et al. 2011), we advise that multiple storage media are used until a pilot study has helped in deciding the most promising method.

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## REFERENCES

Albaugh GP, Iyengar V, Lohani A et al. 1992. Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers.Intl J. Cancer. 52: 347-350.

Banks SC, Piggott, MP, Hansen, BD, Robinson, NA, Taylor, AC, 2002. Wombat coprogenetics: enumerating a common wombat population by microsatellite analysis of faecal DNA. Aust. J. Zool. 50: 103-204.

Bayes MK, Smith KL, Alberts SC, Altmann J, Bruford MW, 2000.Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. Con. Gen. 1: 173–176

Bensch S, Andren, H, Hansson, B, Pedersen, H-C, Sand, H, Sejberg, D, Åkesson, M, Wabakken, P, Liberg, O, 2006. Selection for heterozygosity gives hope to a wild population of inbred wolves. PLoS ONE 1(1): e72. doi:10.1371/journal.pone.0000072.

Bhagavatula J, Singh L, 2006. Genotyping faecal samples of Bengal tiger Panthera tigris tigris for population estimation: a pilot study. BMC Genetics. 7:48.

Bidlack AL, Reed SE, Palsbøll PJ, Getz WM, 2007. Characterization of a western North American carnivore community using PCR-RFLP of cytochrome b obtained from faecal samples. Cons. Gen 8:1511-1513.

Crick HQP, Marshall, PJ, 1981. The birds of Yankari Game Reserve, Nigeria: their abundance and seasonal occurrence. Malimbus 3: 103-114.

Ernest HB, Penedo, MCT, May, BP, Syvaren, M, Boyce, WM, 2000. Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. Mol. Ecol. 9: 433-441.

Ferrando A, Lecis R, Domingo-Roura X, Ponsa M, 2008.Genetic diversity and individual identification of reintroduced otters (*Lutra lutra*) in north-eastern Spain by DNA genotyping of spraints. Con. Gen. 9: 129-139.

Fernando P, Pfrender ME, Encalada SE, Lande R, 2000. Mitochondrial DNA variation, phylogeography and population structure of

the Asian elephant. Heredity 84: 362-372.

Foran DR, Crooks, KR, Minta, SL, 1997. Species identification from scat: an unambiguous genetic method. Wildl. Soc. Bull. 25: 835-839.

Frantz AC, Pope LC, Carpenter PJ et al. 2003. Reliable microsatellite genotyping of the Eurasian badger (Meles meles) using faecal DNA. Mol. Ecol. 12: 1649-1661.

Frantzen MAJ, Silk, JB, Ferguson, JWH, Wayne, RK, Kohn, MH, 1998. Empirical evaluation of preservation methods for faecal DNA. Mol. Ecol. 7: 1423 -1428.

Friedberg EC 2000. DNA damage and repair. Nature 421: 436- 440.

Geerling C, 1973. The vegetation of Yankari Game Reserve, its utilization and condition. Bulletin 3: Department of Forestry, University of Ibadan.

Gerloff U, Schlötterer C, Rassmann et al. 1995. Amplification of hypervariable simple sequence repeats (microsatellits) from excremental DNA of wild living Bonobos (*Pan paniscus*). Mol. Ecol. 4: 515-518.

Gobush K, Kerr B, Wasser S, 2009. Genetic relatedness and the disrupted social structure in a poached population of African elephants. Mol. Ecol. 18:722-734.

Green AA, 1988. Avifauna of Yankari Game Reserve, Nigeria: New records and observations. Malimbus 11: 61-72.

Höss M, Kohn M, Knauer F, Schröder W, Pääbo S, 1992. Excrement analysis by PCR. Nature 359: 199.

Idaghdour Y, Broderick D, Korrida A, 2003. Faeces as a source of DNA for molecular studies in a threatened population of great bustards. Con. Gen. 4:789-792

Kohn M, Wayne RK, 1997. Facts from faeces revisited. TREE 12: 223-227.

Kohn M. Knauer HF, Stoffella A, Schröder W, Pääbo S, 1995. Conservation genetics of the European brown bear- a study using excremental PCR of nuclear and mitochondrial sequences. Mol. Ecol. 4: 95-103.

Kohn MH, York EC, Kamradt DA, Haught G, Sauvajot RM, Wayne RK, 1999. Estimating population size by genotyping faeces. Proc. R. Soc. Lond. Bull. 266:657-663.

Lampa S, Gruber B, Henle K, Hoehn M, 2008. An optimization approach to increase DNA amplification success of otter faeces. Con. Gen. 9: 201-210.

Lindahl T, 1993. Instability and decay of the primary structure of DNA. *Nature* 362: 709-715

Livia L, Francesca V, Antonella P, Fausto P, Bernardino R, 2007. APCR-RFLP method on faecal samples to distinguish *Martes martes, Martes foina, Mustela putorius and Vulpes vulpes*. Con. Gen. 8: 757-759.

Lucchini V, Fabbri E, Marucco F *et al.* 2002. Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in western Italian Alps. Mol. Ecol. 11: 857-868.

Macdonald DW, 1980. Patterns of scent marking with unique urine and faeces amongst carnivore communities. Symp. Zool. Soc. Lond. 45:107-139.

Maudet C, Luikart G, Dubray D, vonHardenberg A, Taberlet P, 2004. Low genotyping error rates in wild ungulate faeces sampled in winter. Mol. Ecol. Notes. 4: 772-775.

Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton Mk, O'Brien SJ, 1999. A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). Genomics 57: 9-23.

Morin PA, Woodruff DS, 1996. Noninvasive genotyping for vertebrate conservation. In: Molecular Genetic Approaches in conservation (Smith TB & Wayne RK). pp. 298-313. Oxford University Press, Oxford.

Murphy MA, Waits LP, Kendall KC, 2000. Quantitative evaluation of faecal drying methods for brown bear DNA analysis. Wildl. Soc. Bull. 28: 951-957.

Murphy MA, Waits LP, Kendall KC, Wasser SK, Higbee JA, Bogden R, 2002. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv. Gen. 3: 435-440.

Nsubuga AM, Robbins MM, Roeder AD, Morin A, Boesch C, Vigilant L, 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. Mol. Ecol. 13: 2089-2094.

Panasci M, Ballard WB, Breck S, Rodriguez D, Densmore III LD, Wseter DB, Baker RJ,

2011. Evaluation of faecal DNA preservation techniques and effects of sample age and diet on genotyping success. J. Wildl. Mgt. 75(7): 1616-1624

Perez I, Geffen E, Mokady O, 2006. Critically endangered Arabian leopards *Panthera pardus nimr* in Israel: estimating population parameters using molecular scatology. Oryx 40: 295-301.

Piggott MP, 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA. Wildl. Res. 31: 485-493.

Piggott MP, Taylor AC, 2003. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. Austr J. Zool. 51: 341- 355.

Prugh LR, Ritland CE, 2005. Molecular testing of observer identification of carnivore faeces in the field. Wildl. Soc. Bul. 33: 189-194.

Puechmaille SJ, Mathy G, Petit EJ, 2007. Good DNA from bat droppings. *Acta Chiropterologica.* 9: 269-276.

Reed JZ, Tollit DJ, Thompson PM, Amos W, 1997. Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. Mol. Ecol. 6: 225-234.

Reddy PA, Bhavanishankar M, Bhagavatula J, Harika K, Ranjeet SM, Shivaji S, 2012. Improved Methods of Carnivore Faecal Sample Preservation, DNA Extraction and Quantification for Accurate Genotyping of Wild Tigers. DOI:10.1371/journal. pone.0046732.g003.

Regnaut S, Francoise SL, Fumagalli L, 2006. DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations. Con. Gen. 7:449-453.

Rutledge LY, Holloway JJ, Patterson BR, White BN, 2008. An improved field method to obtain DNA for the identification from wolf scat. J. Wildl. Mgt.73 (8) 1430-1435.

Santini A, Lucchini V, Fabbri E, Randi E, 2007. Ageing and environmental factors affect PCR success in wolf (Canis lupus) excremental DNA samples. Mol. Ecol. Notes. doi/10.1111/j.1471- 8286.01829.x.

Stenglein JL, deBarba M, Ausband DE, Waits LP, 2010.Impacts of sampling location within a faeces on DNA quality in two carnivore species. Mol. Ecol. Res.10: 109-114.

Taberlet P, Camarra JJ, Griffin S et al. 1997. Noninvasive genetic tracking of the endangered Pyrenean brown bear. Mol. Ecol. 9: 869-876.

Taberlet P, Waits LP, Luikart G, 1999. Non-invasive genetic sampling : look before you leap. TREE. 14: 323-327.

Tende T, Ottosson U, Hansson B, Åkesson M, Bensch S, 2010. Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling. Afr. J. Ecol. 59: 7-12.

Vallet D, Petit E, Gatti S, Levréro F, Ménard N, 2007. A new 2CTAB/PCI method improves DNA amplification success from faeces of Mediterranean (Barbary macaques) and tropical (lowland gorillas) Primates. Con. Gen. DOI 10.1007/s10592-007-9361-8.

Vynne C, Baker MR, Breuer ZK, Wasser SK, 2011. Factors influencing degradation of DNA and hormones in maned wolf scat. Anil. Con. Doi:10.1111/j.1469-1795.2011.00503.x.

Wasser SK, Houston CS, Koehiler GM, Cadd GG, Fain SR, 1997. Techniques for applications of faecal DNA methods to field studies of Ursids. Mol. Ecol. 6: 1091-1097.

Wasser SK, Keim JL, Taper ML, Lele SR, 2011. The influences of wolf predation, habitat loss, and human activity on the caribou and moose in the alberta oil sands. Front Ecol. Env. 9(10): 546-551.

Zhang H-M, Guo Y, Li D-S, Wang P-Y, Fang S-G, 2009. Sixteen novel microsatellite loci developed for the giant panda (*Ailuropoda melanoleuca*). Conser. Gen. 10: 589-592.

# APPENDIX 1. OVERVIEW OF STUDIES COMPARING DIFFERENT PRESERVATION MEDIA FOR FAECAL SAMPLE DNA

| **Authors (Year)** | **Study species/Climate at study sites** | **Preservation methods** | **Relative success / (% success)** |
|---|---|---|---|
| The present study | Lion (*Panthera leo*)<br>Tropical, dry | i) 95% ethanol<br>ii) Two-step storage*<br>iii) Buffer ASL | Highest (50%)<br>Intermediate (20%)<br>Lowest (30%) |
| Reddy *et al.* 2012 | Tiger (*Panthera pardus*)<br>Temperate, dry | i) Two-step storage*<br>ii) Ethanol<br>iii) Silica beads only | Highest<br>Intermediate<br>I n Lowest |
| Calderon *et al.* 2009 | Forest ungulates (*Cephalophus* spp.)<br>Tropical, humid | i) RNA*later*<br>ii) 95% Ethanol<br>iii) Silica beads only | Highest<br>Intermediate<br>Lowest |
| Santini *et al.* 2007 | Wolf (*Canis lupus*)<br>Temperate, dry | i) i) 95% Ethanol at freezer -20°<br>ii) GUS lysis buffer**<br>iii) Only freezer -20°<br>iv) 95% Ethanol at room temperature | Highest (98%)<br>Second best (92%)<br>Intermediate (71%)<br>Lowest (55%) |
| Nsubuga *et al.* (2004) | Mountain gorilla (*Gorilla beringei beringei*)<br>Chimpanzee (*Pan troglodytes versus*)<br>Tropical, humid | i) Two-step storage*<br>ii) Silica beads only<br>iii) RNA*later* solution | Highest<br>Intermediate<br>Intermediate |
| Roeder *et al.* (2004) | Gorilla (Gorilla gorilla)<br>temperate, dry | i) 90% Ethanol<br>ii) Two-step storage*<br>iii) Silica beads only | Highest<br>Second best<br>Lowest |
| Frantz *et al.* (2003) | Eurasian Badger (*Meles meles*)<br>Temperate | i) 70% Ethanol<br>ii) DETs ***<br>iii) Freezing at -20°C | Highest (89%)<br>Intermediate<br>Lowest |
| Piggot & Taylor 2003 | Tasmanian pademelon (*Thylogale billardierii)*<br>Temperate | i) Freezing at -20°C<br>ii) 70% Ethanol<br>iii) Dried at room temperature<br>iv) DETs buffer*** | High (55%)<br>High (55%)<br>High (55%)<br>Lowest (40%) |

| | | | |
|---|---|---|---|
| Murphy *et al.* (2002) | Brown bear (*Ursus arctos*) Temperate | i) 90% Ethanol<br>ii) Silica beads only<br>iii) Oven-dried (stored at room temperature)<br>iv) Oven-dried (stored at -20°C) | Highest (86%)<br>Intermediate<br>Lowest<br>Lowest |
| Murphy *et al.* (2000) | Brown bear (*Ursus arctos*) Temperate | i) Freeze-drying<br>ii) Oven drying<br>iii) Silica beads only<br>iv) Microwave drying | Highest (98%)<br>Second best (95%)<br>Lowest<br>Lowest |
| Frantzen *et al.* (1998) | Baboons (*Papio cynocephalus ursinus*) Temperate – Sub-tropical | i) DETs buffer***<br>ii) Air-dried at room temperature<br>iii) Frozen at -20°C<br>iv) 70% ethanol | Highest (70%)<br>Second best (67%)<br>Intermediate (60%)<br>Intermediate (61%) |
| Wasser *et al.* (1997) | American black bear (*Ursus americanus*)<br>Sun bear (*Helarctos malayanus*)<br>Temperate, dry | i) Silica beads only<br>ii) Ethanol<br>iii) Frozen at -20°C | Highest<br>Intermediate<br>Lowest |
| Panasci et al. (2011) | Coyote (*Canis latrans*) | i) 95% ethaol<br>ii) DETs buffer***<br>iii) Lysis buffer | High<br>High<br>Lowest |

*Ethanol and silica gel beads
** 3M guanidine thiocyanate, 50 mM Tris-HCl pH 7.0, 25 mM EDTA pH 8.0, 25% Triton X-100
***20% DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5 and NaCl to saturation

# DUAL PHYLOGENETIC ORIGINS OF NIGERIAN LIONS *(PANTHERA LEO)*

III

# DUAL PHYLOGENETIC ORIGINS OF NIGERIAN LIONS *(PANTHERA LEO)*

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## ABSTRACT

Lion faecal DNA extracts from four individuals each from Yankari Game Reserve and Kainji-Lake National Park (Central North East and West Nigeria, respectively) were Sanger sequenced for the mitochondrial cytochrome *b* gene. The sequences were aligned against 61 lion reference sequences from other parts of Africa and India. The sequence data was analyzed further for the construction of phylogenetic trees using the maximum likelihood approach to depict phylogenetic patterns of distribution amongst sequences. Our results show that Nigerian lions grouped together with lions from West and Central Africa, including India. However, at the smaller geographical scale, lions from Kainji-Lake National Park in western Nigeria grouped with lions from Benin (located west of Nigeria), whereas lions from Yankari Game Reserve in central north-eastern Nigeria grouped with the lion populations in Cameroon (located east of Nigeria). The finding of two lion populations of different phylogenetic origins in this study is important for future management and conservation decisions for lions in Nigeria, Benin and Cameroon.

**Keywords:** Dual origins, Nigerian lions, Faecal DNA, Sanger sequencing.

# INTRODUCTION

There have been recent reports about a rapid reduction in population size and range distribution of lions (Estes et al. 2011; Packer et al. 2013). Until rather recently, lions were broadly distributed and roamed most parts of southern Europe, Asia, the Middle East, North America, northern part of South America and sub Saharan Africa (Cohelach 1982; Turner and Anton 1997; Bauer and Van der Merwe 2004; Werdelin and Lewis 2005). They were believed to have had the widest geographical distributions of any large terrestrial mammal in the late Pleistocene (Guthrie 1990; Kitchener 1991; Nowell and Jackson 1996; Sunquist and Sunquist 2002; Patterson 2004; Barnett et al. 2009) before their disappearance as part of the end-Pleistocene mega faunal mass extinction (Martin and Steadman 1999). Today wild lions are found only in some parts of sub Saharan Africa and at one locality in India, where they are confined mainly to supposedly protected areas such as national parks and game reserves. Even the relict populations found in these places seem to be declining at an alarming rate due to anthropogenic activities (Smuts 1978; Hanby and Bygott 1979; Nowell and Jackson 1996; Martin and Steadman 1999). Lions are today classified as vulnerable according to the IUCN Red List of Threatened Species (www.iucnredlist.org). Their range collapse in historic times must have been responsible for the elimination of most and especially very marginal populations (O'Brien et al. 1987; Kingdon 1997).

All Pleistocene and modern day lions have been assigned to the genus *Panthera*, but with little consensus about the extent of overlap in their distribution (Barnett et al. 2009). Just like other big cats (e.g. leopard *Panthera pardus*, tiger *Panthera tigris* and jaguar *Panthera unca*), the lion displays several distinct phenotypic variations in body size, skull characteristics, coat colour and thickness, retention of juvenile spots and presence or absence of mane in males. These marked characteristics sometimes may vary based on geographical regions (Hallgrimsson and Maiorana 2000; Mazak 2010). Many studies have employed the method of comparative analysis of craniometric data and morphometric analysis based on geographic regions to establish phylogenetic relationships between the lions (Sotnikova and Nikolskiy 2006; Mazak 2010). These analyses are then used in establishing distinctiveness between geographical regions (Hallgrimsson and Maiorana 2000; West and Packer 2002; Patterson 2004; Yamaguchi et al. 2004; Patterson et al. 2006; Patterson 2007). But there can be complications with morphological identification sometimes due to the presence of shared primitive features (Sotnikova and Nikolskiy 2006), where morphological characteristics might not depict the true phylogeny of a species.

Different names have been proposed for the African lion based on geographic race (Meester and Setzer 1971). Taxonomic authorities recognise only two subspecies of lion; the African lion (*P. leo leo*, Linneaus 1758*)* and the Asian lion (*P. leo persica*, Meyer 1826) (O'Brien et al. 1987), and this has been supported by different genetic studies (e.g. O'Brien et al. 1987; Driscoll et al. 2002; Burger et al. 2004; Dubach et al. 2005; Barnett et al. 2006). Many of these previous genetic studies did not have a good representation of lions from all over their range in West and Central Africa. A recent phylogeographical study by Bertola et al. (2011) which was based on mitochondrial DNA sequences had good representation of lions form West and Central Africa. They found that Indian lions clustered with West and Central African lions. Although their study had a good representation of lions from West and Central Africa, they did not include lions from some parts of West Africa – most notably Nigeria.

In Nigeria, lions remain today in two isolated populations only, one in Kainji-Lake National Park in the western part of the country and one in Yankari Game Reserve in central north-east (Fig. 1). The phylogenetic relationship between the lion populations in Nigeria and elsewhere in West and Central African is not yet fully understood. It is important to know, in the context of conservation, if the lion populations in Nigeria form a monophyletic clade, or if the two populations within Nigeria form separate clades. Describing the genetic makeup and phylogeographic history of endangered species is important for understanding the evolutionary processes affecting the species and their geographical dynamics. This is important for developing conservation strategies and can thus aid in making future management decisions easier (Mace et al. 2003).

An on-going survey by Tende et al. (*In press*) to determine the population size and level of gene flow within and between the lion populations in Nigeria has shown that the two remaining populations in Kainji-Lake National Park and Yankari Game Reserve exhibit signs of inbreeding and that they are genetically differentiated. In the present study, we aim to find out the relationship of the Nigerian lions to the closely located populations from the neighbouring countries, as well as to lions in other parts of Africa and Asia, by phylogenetic analysis of the mitochondrial cytochrome *b* gene.

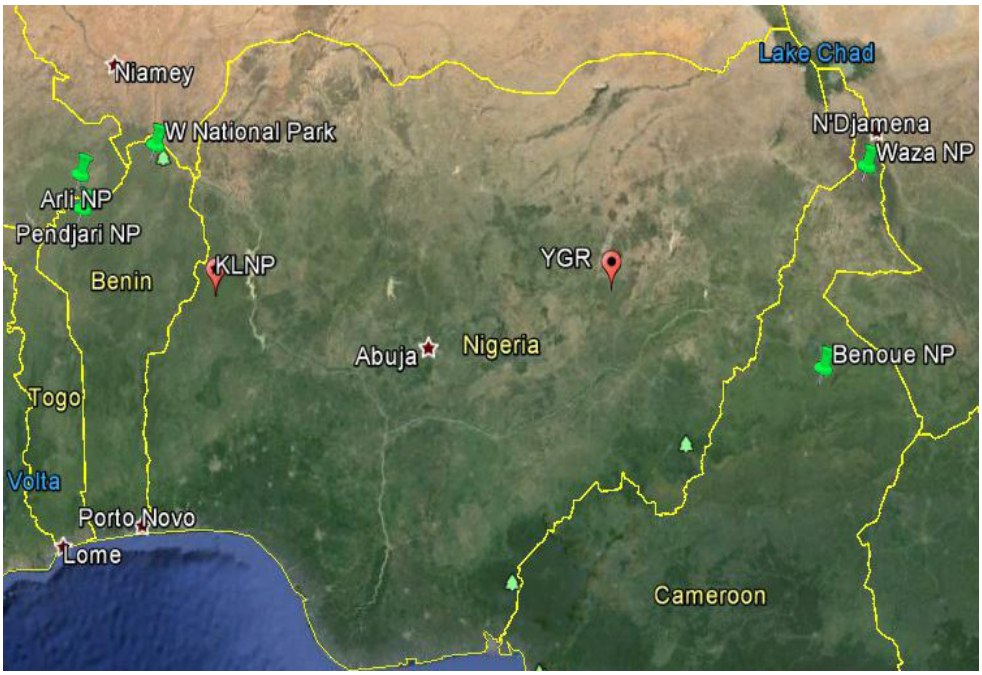


**Figure 1.** Locations of the two lion populations in Nigeria, KLNP and YGR (indicated by red markers), and neighbouring lion populations in Benin and Cameroon (indicated by green markers).

## MATERIALS AND METHODS

Lion faecal DNA extracts from four different supposedly unrelated individuals from each of the study sites Yankari Game Reserve, Central North East (9° 50'N and 10° 30'E) and Kainji-Lake National Park, West Nigeria (09° 55'N 03° 57'E) were identified based on genotypes of nine microsatellite loci (Tende et al. *in press*). Details of the DNA extraction protocol are given in Tende et al. (2010). Primers were designed to amplify three different segments covering most of the 1140 base pairs of the mitochondrial cytochrome *b* region. The primer sets for these regions are as follows: LCB1F (5'-TCACCGGCCTCTTTCTAGCCA-3') and LCB1R (5'-AGGTGGACTGCTGCTAGGGCT-3'), LCB2F (5'-TCGGGGCCGACCTAGTAGAGTG-3') and LCB2R (5'-TGGAAGTGTGGAGGGCAGGGA-3'), and LCB3F (5'-CCCGACAACTATACCCCCGCCA-3') and LCB3R (5'-AGGGTACGCGTTCTCCTTTT-3'). All amplifications were carried out using a 2X Qiagen multiplex PCR kit in 10 µl reaction volume containing 5 µl Qiagen multiplex PCR buffer mix, 0.2 µM forward primers (Applied Biosystems), 0.2 µM reverse primer, 2.6 µl of water and 2 µl of DNA extract. A hot start at 95°C for 15 minutes with PCR profiles consisting of 35 cycles as follows: 90°C for 30 s; annealing temperature of 56°C for 30 s with elongation period of 72°C for 30 s. A blank control (reagents only) from extracted DNA process was included in all PCRs to monitor for contamination. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining. Samples were further sequenced using the forward primers (BigDye sequencing kit; Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems). Sequences were visually checked and aligned using Geneious vs.5.6.6 against 61 lion reference sequences from other parts of Africa and India downloaded from the Genbank.

The program MEGA5 (Tamura *et al*. 2011) was used to analyse the sequence data for the construction of phylogenetic trees. The substitution model for the construction of the tree was selected based on the lowest Bayesian information criterion (BIC). We identified the Hasegawa-Kishino-Yano (HKY) model as the best to describe the substitution pattern (Nei & Kimura, 2000). The statistical confidence of each node was determined by assessing the frequency of nodes supported in 1000 bootstrap resampling of our data (Felsenstein 1985).

We used the maximum likelihood approach with leopard (*Panthera pardus*) and tiger (*Panthera tigris*) as out-groups for the mitochondrial cytochrome b sequences to depict phylogenetic patterns amongst sequences.

## RESULTS

We obtained sequences for all eight samples covering 944 bp of the cytochrome *b* gene. The analysed samples showed no sequence variation within the two Nigerian study sites but differed by 0.4 % between these sites. The cytochrome *b* phylogenetic analysis showed that the Nigerian lions cluster with 96% bootstrap support with lions from West and Central Africa and India (Fig. 2). Interestingly, the two Nigerian populations were phylogenetically separated: lions from Kainji-Lake National Park in western Nigeria grouped with lions in Benin (with 83% bootstrap support), whereas the population in Yankari Game Reserve in central north-eastern Nigeria grouped with lions from Cameroon and a few other countries (92% bootstrap; Fig. 2).

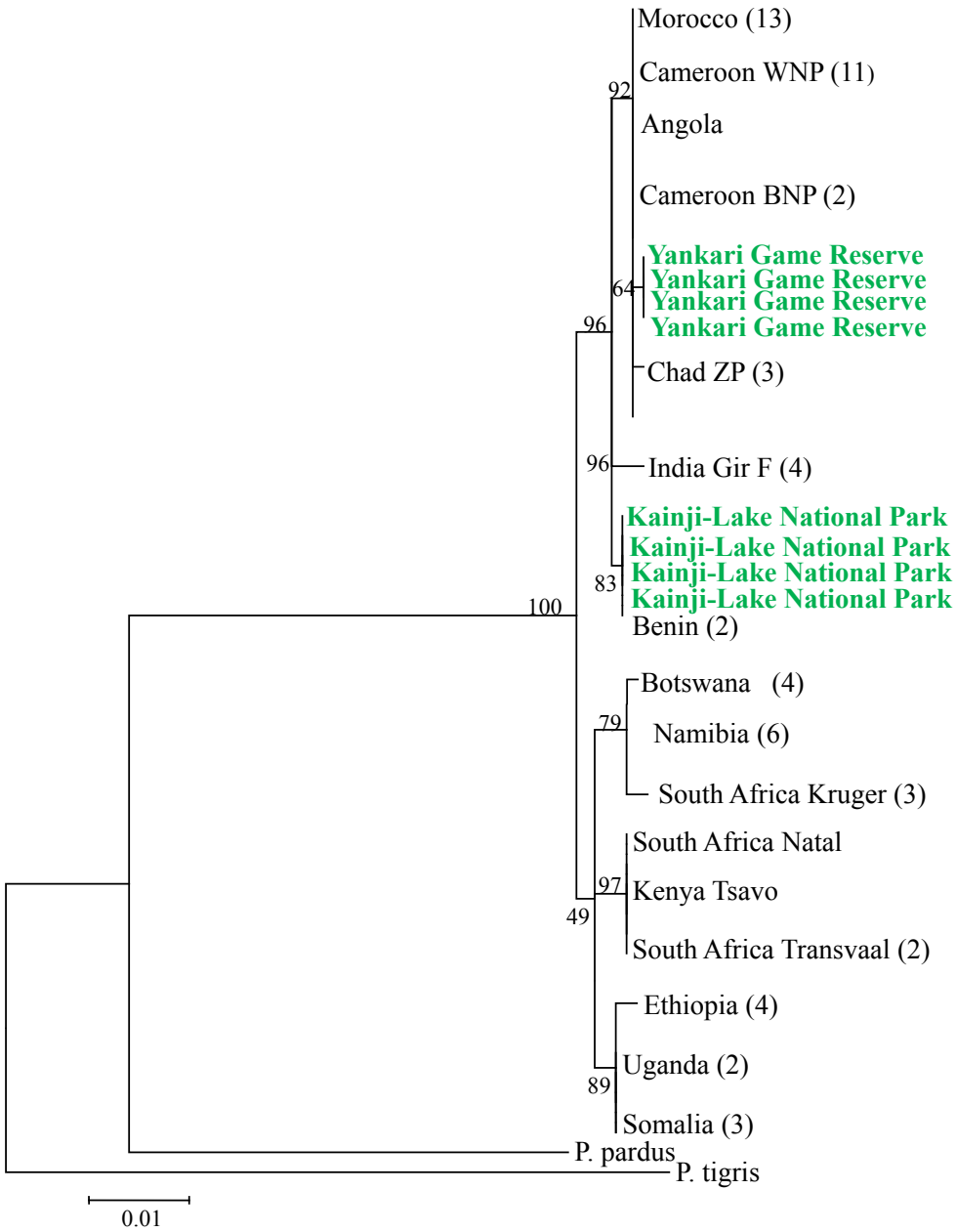


**Figure 2.** Phylogenetic tree from a maximum likelihood analysis based on a set of lion mitochondrial cytochrome *b* sequences. Numbers in bracket represent the number of lion sequences downloaded from the genbank for each area. Abbreviations are as follows: Cameroon BNP (Cameroon Benuoe National Park), Cameroon WNP (Cameroon Waza National Park), Chad ZP (Chad Zakouma National Park), SA Transvaal (South Africa Transvaal), SA Kruger (South Africa Kruger National Park). Highlighted in green are individuals from Yankari Game Reserve and Kainji-Lake National Park in Nigeria.

# DISCUSSION

Using molecular data to reveal the distribution of genetic variation within and between populations can be powerful means to enhance our understanding about the evolutionary history of populations. For instance, Bertola et al. (2011) analysed the cytochrome *b* gene and the control region of the lion from most parts of its range. They found that lions in West and Central Africa group with Asian lions rather than Southern and Eastern African populations. Our results, although restricted to the cytochrome *b* gene, are in agreement with their findings.

Our main finding is that lions from the two remaining populations in Nigeria do not cluster as sister taxa in phylogenetic mtDNA reconstructions. Instead, the cytochrome *b* gene of lions from Kainji-Lake National Park in western Nigeria are genetically similar to lions in Benin, whereas lions from Yankari Game Reserve in central north-eastern Nigeria are more similar to the Cameroon lion population. This phylogeographic division probably reflects long-term separation due to large spatial geographic distance between these two localities and lack of dispersal corridors for lions in Nigeria. In fact, the localities are geographically closer to populations in different neighbouring countries; Benin in the case of KLNP and Cameroon in the case of YGR. The population in Kainji-Lake National Park may share a common history with lions in the nearby WAP complex, i.e. the W and Pendjari National Parks in Benin, and Arly National Park in Burkina Faso (Fig.1).

Recent surveys have shown drastic declines in population size and range of the lions (Packer et al. 2013). A recent survey on lions in Nigeria has shown that the population is small with low genetic variability (Tende et al. in press). As lions increasingly get confined to supposedly protected areas, the strategy for meta-population management will involve moving individuals between areas for population recovery and genetic reinforcement. The exchange of individuals between the two unique lion populations in Nigeria could help to enhance their different gene pool. Also translocation can be carried out between the remaining relict lion populations in Nigeria and neighbouring countries of Benin and Cameroon when there is a need for that. Various studies have shown how natural exchanges of few individuals between populations have helped to enhance population growth and restore genetic diversity (Laikre and Ryman 1991; Liberg et al. 2005; Vila et al. 2003). For instance, low population size and genetic diversity were recorded among the Scandinavian wolves before the population was rescued by a single immigrant from Finland (Vila et al. 2003). The arrival of this immigrant into the Scandinavian wolf population provided the possibility to avoid inbreeding, decrease the risk of inbreeding depression and resulted in population growth. Also laboratory and translocation experiments have indicated that small and inbred populations can be rescued by the contribution of minimal number of immigrants, helping to decrease inbreeding depression (Spielman and Frankham 1992; Westemeier et al. 1998; Madsen et al. 1999; Ebert et al. 2002; Vila et al. 2003), and bring about profound changes in genetic structures (Ball et al. 2000; Saccheri et al. 2002; Vila et al. 2003). But before a successful translocation program can be achieved, it is recommended that lions that are used should have the same genetic make-up in order to avoid introduction of genes from distant populations.

**Dispersal difference in male and female lions**

In vertebrates, mtDNA population genetic analyses is confined to tracing the migration patterns of maternal lineages, while analysis of the nuclear DNA inherited through

both parents may give a complete picture of population structure inherited from both parents. Thus being maternally inherited, mtDNA population genetic structures would reflect maternally-directed natal site fidelity and gene flow, whereas genome wide biparentally inherited nDNA assists in quantifying the levels of gene flow among subpopulations for both sexes. High rate of male-biased difference in dispersal patterns in lions (Pusey and Packer 1987) are expected to result in different distributions of genetic variation among populations for maternally (mtDNA) versus biparentally (nDNA) inherited molecular markers. The fixed differences in mtDNA between Kainji and Yankari lions suggest that female mediated gene flow between the parks have been small or absent. Our study based on microsatellite loci (nDNA) to investigate genetic differentiation between the two populations also found substantial population structure ($F_{ST}$=0.17) suggesting low levels of gene flow (Tende et al. *in press).* The suggested absence of female migration between our two study sites agrees with field studies demonstrating strongly restricted female dispersal in lions. Females can leave their natal pride to establish a new pride adjacent to their natal range which often includes part of their old range as compared to males that can disperse long distances from their natal range (Pusey and Packer 1987, Spong and Creel 2001). But whether the difference in mtDNA reflects overall differences in nuclear DNA in this study needs further investigation. The observed differences between the two populations could be due to phylogeography or genetic drift; a random change in allele frequency caused by chance event in small population sizes. The smaller the population size, the more likely genetic drift is to occur due to sampling errors.

**Conclusion and conservation perspectives**

In summary, phylogenetic analysis of 944 base pairs of mitochondrial sequence data suggests that lions in Kainji-Lake National Park in western Nigeria are more closely related to lions in Benin, whereas lions in Yankari Game Reserve in central north-eastern Nigeria are more closely related to lions in Cameroon. This difference reveals little or absence of female-mediated gene flow between the two Nigerian populations. Our finding is relevant for the management of the West and Central African lions, and serves as an important guide for future conservation and management decisions. This study has shown the genetic makeup of the Nigerian lion and their relationship to lions in the two neighbouring West and Central African countries.

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**Conflict of interest**

None declared

## REFERENCES

Ball, S. J., M. Adams, H. P. Possingham, and M. A. Keller. 2000. The genetic contribution of single male immigrants to small, inbred populations: a laboratory study using Drosophila melanogaster. Heredity 84: 677-684.

Barnett, R., N. Yamaguchi, I. Barnes, and A. Cooper. 2006. The origin, current diversity,

and future conservation of the modern lion(*Panthera leo*). Proceedings of the Royal Society Of London Bulletin 273: 2159-2168.

Barnett, R., B. Shapiro, I. Barnes, S. Y. W. Ho, J. Burger, N. Yamaguchi, G. Higham, H. T. Wheeler, W. Rosendahl, A. V. Sher, M. Sotnikova, T. Kuznetsova, G. Baryshnikov, L. Martin, R. Harington, J. Burns, A. Cooper.2009. Phylogography of lions (Panthera leo ssp.) reveals three distinct taxa and a late Pleistocene reduction in genetic diversity. Molecular Ecology 18:1-10.

Baryshnikov, G. F., and G. Boeskorov. 2001. The Pleistocene cave lion, Panthera spelaea (Carnivora,Felidae) from Yakutia, Russia. Cranium 18: 7-24.

Bauer, H., and S. Vander Merwe. 2004. Inventory of free ranging lions *Panthera leo* in Africa. Oryx 38: 26-31.

Bertola, L. D., W. F. Van Hooft, K. Vrieling, D. R. Uit de Weerd, D. S. York, H. Bauer, H. H. T. Prins, P. J. Funston, H. A. Udo de Haes, H. Leirs, W. A. VanHaeringen, E. Sogbohossou, P. N. Tumenta, and H. H. De Iongh. 2011. Genetic diversity, evolutionary history and implications for conservation of the lion (*Panthera leo*) in West and Central African Journal of Biogeography. 38:1356 -1367.

Bensch, S., H. Andren, B. Hansson, H-C. Pedersen, H. Sand, D. Sejberg, P. Wabakken, M. Åkesson, and O. Liberg. 2006. Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS ONE* 1(1):e72.doi:10.1371/journal.pone.0000072.

Burger, J., W. Rosendahl, O. Loreille, H. Hemmer, T. Eriksson, A. Gotherstrom, J. Hiller, M. J. Collins, T. Wess, and K. W. Alt. 2004. Molecular phylogeny of the extinct cave lion *Panthera leo spelaea*. Molecular Phylogenetics and Evolution 30: 841-849.

Coheleach, G. 1982. The Big Cats: The Paintings of Guy Coheleach. Harry N. Abrams, Inc., New York.

Driscoll, C. A., M. Menotti-Raymond, G. Nelson, D. Goldstein, and S. J. O'Brien. 2002. Genomic microsatellites as evolutionary chronometers: a test in wildcats. Genome Research 12: 414-423.

Dubach, J., B. D. Patterson, M. B. Briggs, K. Venzke, J. Flamand, P. Stander, L. Scheepers, and R. W. Kays. 2005. Molecular genetic variation across the southern and eastern geographic ranges of the African lion. Conservation Genetics 6: 15-24.

Ebert, D., C. Haag, M. Kirkpatrick, M. Riek, J. W. Hottinger, and V. I. Pajunen. 2002. A selective advantage to immigrant genes in a Daphnia meta population. Science 295:485- 488.

Estes, J. A., J. Terborgh, J. S. Brashares, M. E. Power, J. Berger, W. J. Bond, S. R. Carpentar, T. E. Essington, R. D. Holt, J. B. C. Jackson, R. J. Marquis, R. Oksanen, T. Oksanen, R. T. Paine, E. K. Pikitch, W. J. Ripple, S. A. Sandin, M. Scheffer, T. W. Schoener, J. B. Shurin, A. R. E. Sinclair, R. V. Soule, and D. A. Wardle. 2011. Trophic downgrading of planet earth. Science 333: 301

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39: 783-791.

Guthrie, R. D. 1990. Frozen Fauna of the Mammoth Steppe: The Story of Blue Babe. University of Chicago Press, Chicago.

Guggisberg, C. A. W. 1961. Simba, the Life of the Lion. Howard Timmins, Capetown, SouthAfrica.

Hallgrimsson, B., and V. Maiorana 2000. Variability and size in mammals and birds. Biological Journalof the Linnean Society 70:571-595.

Hanby, J. P., and J. D. Bygott 1979. Population Changes in Lions and Other Predators. In Serengeti: Dynamics of an Ecosystem.(eds. Sinclair ARE and Norton Griffiths M), pp.249-262. University of Chicago Press, Chicago.

Kingdon, J. 1997. The Kingdon Field Guide to African Mammals. Academic Press, San Diego,California.

Kitchener, A. C. 1991. The natura lhistory of wildcats. New York: Cornell University Press.

Laikre, L., and N. Ryman. 1991. Inbreeding depression in a captive Wolf (*Canis lupus*) population. Conservation Biology 5: 33- 34

Liberg, O., H. Andren , C. H. Pedersen, H. Sand, D. Sejberg, P. Wabakken, M. Åkesson, and S. Bensch. 2004. Severe inbreeding depression in a wolf (*Cani lupus)* population. Biology

Letters (xxxx) doi:10.1098/rsbl.2004.0266

Mace, G. M., J. L. Gittleman, and A. Purvis. 2003. Preserving the tree of life. Science 300: 1707–1709.(doi:10.1126/science.1085510)

Madsen, T., R. Shine, M. Olsson, and H. Wittzell.1999. Conservation biology: restoration of an inbred adder population. Nature 402:34-35.

Martin, P. S., and D. W. Steadman. 1999. 2. Prehistoric extinctions on islands and continents. In: Extinctions in Near Time: Causes, Contexts, and Consequences (ed. MacPhee RDE), pp. 17-50. Kluwer Academic/Plenum Publishing, New York.

Mazak, J. H. 2010. Geographical variation and phylogenetics of modern lions based on craniometric data. Journal of Zoology 281:194-209.

Meester, J. A. J., H. W. Setzer. 1971. The Mammals of Africa. An Identification Manual. Smithsonian Institution, Washington,D.C.

Nei, M., and Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nowell, K., and P. Jackson. (Compilers and editors) (1996) Wild cats: Status survey and Conservation Action Plan. IUCN/ SSC Cats Specialist Group. IUCN, Gland, Switzerland.

O'Brien, S. J., J. S. Martenson, C. Packer, L. Herbst, V. D. Vos , P. Joslin, J. Ott-Joslin , et al. 1987. Biochemical genetic variation in geographic isolates of African and Asiatic lions. National Geographic Research 3:114-124.

Packer, C., A. Loveridge, S. Canney, T. Caro, et al. 2013. Conserving large carnivores: dollars and fence. Ecology Letters Doi:10.1111/ ele.12091.

Patterson, B. D. 2004. The Lions of Tsavo: Exploring the legacy of Africa's Notorious Maneaters. McGraw-Hill, New York.

Patterson, B. D. 2007. On the nature and significance of variability in lions (*Panthera leo*). Evolutionary Biology 34: 55-60.

Patterson, B. D., R.W. Kays, S. M. Kasiki, and V. M. Sebestyen. 2006. Developmental effects of climate on the lion's mane (*Panthera leo*). Journal of Mammology 87:193-200.

Pusey, A. E., and C. Packer. 1987. The evolution of sex-biased dispersal in lions. Behaviour 101: 275-310.

Saccheri, I. J., and P. M. Brakefield. 2002. Rapid spread of immigrant genomes into inbred populations. Proceedings of the Royal Society of London Bulletin 269:1073-1078.

Smuts, G. L. 1978. Effects of population reduction on the travels and reproduction of lions in Kruger National Park. Carnivore 1:17-28.

Sotnikova, M., and P. Nikolskiy. 2006. Systematic position of the cave lion Panthera spelaea (Goldfuss) based on cranial and dental characters. Quaternary International 142-143, 218-228.

Spielman, D., and R. Frankham. 1992. Modelling problems in conservation genetics using captive Drosophila populations: improvement of reproductive fitness due to immigration of one individual into small partially inbred populations. Zoo Biology 11:343-351.

Spong, G., and S. Creel 2001. Deriving dispersal distances from genetic data. Proceedings of the Royal Society of London Bulletin 268: 2571-2574.

Sunquist, M., and F. Sunquist. 2002. Wild cats of the world. Chicago: University of Chicago Press.

Tamura, K., D. Peterson., N. Peterson., G. Stecher., M. Nei., and S. Kumar (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28: 2731- 2739.

Tende T, Ottosson U, Hansson B, Åkesson M, Bensch S, 2010. Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling. African Journal of Ecology 59: 7-12.

Turner, A., and M. Anton. 1997. The Big Cats and their Fossil Relatives. Columbia University Press, NewYork. 234pp.

Vereshchagin, N. K. 1971. The cave lion and its history in the Holarctic and on the territory of the U.S.S.R.Akademia Nauk SSSR, Zological Institute Trudy 49:123-199.

Vila, C., A. K. Sundqvist, and O. Flagstad. 2003. Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. Proceedings of the Royal Society Bulletin 270: 91-97.

Werdelin, L., and M. E. Lewis.2005. Plio-Pleistocene Carnivora of eastern Africa: species richness and turnover patterns. Zoological Journal of the Linnean Society. 144:121-144.

West, P. M., and C. Packer. 2002. Sexual selection, temperature, and the lion's mane. Science, 297:1339–1343.

Westemeier, R. L., J. D. Brawn, S. A. Simpson, T. L. Esker, R. W. Jansen, J. W. Walk, E. L. Kershner, J. L. Bouzat, and K. N. Paige. 1998. Tracking the long-term decline and recovery of an isolated population. Science 282: 1695-1698.

Yamaguchi, N., A. Cooper, L. Werdelin, and D. W. Macdonald. 2004. Evolution of the mane and group-living in the lion (*Panthera leo*): a review. Journal of Zoology London 263:329-342.

# POPULATION SIZE OF LIONS IN YANKARI GAME RESERVE AS REVEALED BY FAECAL DNA SAMPLING

IV

African Journal of Ecology

# Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling

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## Abstract

Studies have shown that lion (*Panthera leo*) populations in West Africa are small, isolated and fragmented. In Nigeria, lions have disappeared from unprotected areas and are nowadays found only in parks and reserves where these populations may still decline. It is therefore urgent to obtain reliable estimates of population sizes at different localities. Direct observational surveys may either fail to count all individuals or count some individuals repeatedly and are therefore associated with unknown levels of estimation errors. More accurate estimates can be obtained if direct counting is combined with DNA-based individual identification. As lions are difficult to identify individually, presented here is a method that can be a valuable addition to the existing census methods.

*Key words:* African lion, faecal DNA, isolated population, noninvasive sampling

## Résumé

Des études ont montré que les populations de lions *Panthera leo* d'Afrique de l'Ouest sont petites, isolées et fragmentées. Au Nigeria, les lions ont disparu des zones non protégées et ne se trouvent plus aujourd'hui que dans des parcs et des réserves où leurs populations peuvent d'ailleurs encore décliner. Il est donc urgent d'avoir des estimations fiables de la taille des populations en différents endroits. Les études par observations directes risquent de ne pas réussir à compter tous les individus ou, à l'opposé, d'en compter certains plusieurs fois, et on les associe dès lors de niveaux d'erreur d'estimation inconnus. On peut obtenir des estimations plus précises si les comptages directs sont combinés avec des identifications individuelles basées sur des analyses d'ADN. Comme les lions sont difficiles à identifier individuellement, voici présentée ici une méthode qui peut être un ajout intéressant aux méthodes de recensement existantes.

## Introduction

The population size of many species, including several large African mammals, has declined drastically in recent years, most often because of anthropogenic activities (e.g. Nowell & Jackson, 1996). To be able to conserve long-term viable populations, it is urgent to get reliable estimates of population sizes at different localities. Direct counts through observations provide reliable estimates of population sizes for species where most individuals are encountered, but, for secretive species and/or in dense habitats, this method may fail to provide an accurate estimate. A DNA-based analysis of faeces (Höss *et al.*, 1992) is potentially a reliable method of estimating population sizes (Kohn & Wayne, 1997; Kohn et al., 1999). This method requires that faeces can be easily found and recovered (Kohn *et al.*, 1999), and is well suited for large carnivores such as lions (*Panthera leo*), which often defecate along trails to mark territory boundaries (Macdonald, 1980). This method has been successfully used in studies on e.g. mountain lions (*Puma concolor*) in California (Ernest *et al.*, 2000) and wolves (*Canis lupus*) in Scandinavia (Bensch *et al.*, 2006). Taberlet & Luikart (1999) recommended that any study requiring the use of noninvasive genetic method should be preceded by a pilot study to assess the feasibility and reliability of the method, e.g. error rates and difficulties such as allelic drop-outs and false alleles that might be encountered during genotyping DNA.

In this pilot study, the use of faecal samples for estimating the population size and genetic status of lions in Yankari

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Game Reserve, one of the few protected areas in Nigeria which still harbours lions, is evaluated. The total population of lions in Nigeria has been estimated to be between 100 and 300 individuals (Bauer & Van Der Merwe, 2004).

## Materials and methods

This study was carried out in Yankari Game Reserve (The Reserve), 9°50′N and 10°30′E, located 100 km south east of Bauchi town in Bauchi state. The Reserve is situated on the border of the Sudan – Guinea Savannah zone and covers an area of 2244 km$^2$ (Geerling, 1973). The vegetation is made up of swampy flood plains bordered by patches of forest (gallery and riparian), woodland savannah and human occupation zones (farmland and villages; Crick & Marshall, 1981; Green, 1989).

The collection of faecal samples was carried out systematically along predetermined tracks in the Reserve. Intensive searches took place daily in the core area of the Reserve, along the River Gaji covering about 125 km$^2$ (Fig. 1). A total of 356 faecal samples were collected. All faecal samples were stored in 99% ethanol and after arrival at the laboratory stored at −50°C until DNA extraction. DNA was extracted from faeces using the Stool DNA extraction protocols in QIAamp® (Qiagen, Valencia, CA, USA). For reasons of time and money constraints, DNA extraction was restricted to include 108 samples only. The PCR-technique was used to amplify a short (206 bp) portion of the mitochondrial *cytochrome b* from the extracted samples. The

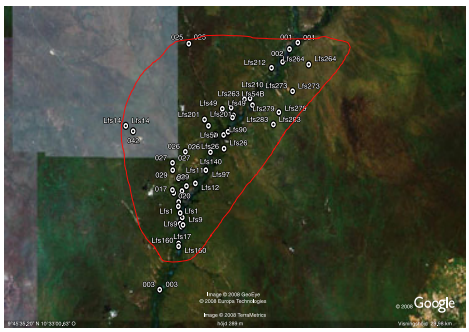


**Fig 1** Google Earth image with observations, 001–042, and sites for faecal sampling points marked Lfsxxx. The red line shows the area within which all observations and faecal sample collections were carried out, an area of about 125 km$^2$

primers LIHYF (5′-ATGACCAACATTCGAAAATCWC-3′) and LIHYR (5′-ATGTGGGTSACTGATGAG-3′) were designed to avoid amplification of human and ungulate DNA in general to promote detection of the target species. All amplifications were performed using AmpliTaq Gold (Perkin Elmer, San Jose, CA, USA) with a hot start at 94°C for 10 min in a reaction volume of 25 μl. PCR profiles consisted of 35 cycles as follows: 90°C for 30 s; annealing temperature of 52°C for 30 s with elongation period of 72°C for 30 s. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and ethidium bromide staining. Positive samples were sequenced using the forward primer (BigDye sequencing kit; Applied Biosystems, Foster City, CA, USA) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems).

### *Microsatellites*

Primers to amplify two polymorphic microsatellites, Ple53 and Ple56, came from Gaur *et al.* (2006). The PCR-mix contained 0.4 μM dye-labelled (6-Fam or Hex) F-primer, 0.4 μM R-primer, 15 nmol $MgCl_2$, 1.25 nmol dNTP, 0.5 U AmpliTaq GOLD polymerase and 2 μl DNA in a 10 μl reaction. PCRs were performed using a GeneAmp 9700 thermocycler (Applied Biosystems): 94°C for 15 min; 35 cycles at 94 °C 30 s, 62/54°C 30 s for Ple53/Ple56, and 72°C 45 s; followed by 72°C for 10 min. PCR products were separated on 6% polyacrylamide gels and alleles detected in a Typhoon 9200 (Amersham Biosciences, São Paulo, Brazil). Poor quality of DNA in faecal samples makes the analyses prone to result in allelic drop-outs (i.e. only one of the alleles in heterozygote individuals amplify). To accommodate this problem in our analyses, all samples were amplified at each locus three times for confirmation.

## Result and discussion

In total, 356 faecal samples were collected from the central part of the Reserve (Fig. 1). From a subset of 108 samples, DNA from 43 faecal samples (40%) was successfully amplified. This proportion is a little bit less than in a study on mountain lions where they found DNA in 47% of the samples (Ernest *et al.*, 2000) and much less than in a study on snow leopards (*P. uncia*), 72–89% (Janečka *et al.*, 2008). When tested with BLAST against the GenBank sequence data base, 33 mitochondria *cytochrome b* sequences matched with the lions and two with the spotted hyaena (*Crocuta crocuta*). For the microsatellite

analyses, only the 33 samples confirmed to contain lions DNA (see below) was used.

To obtain individual molecular fingerprints, the 33 lion samples were scored for allelic variation at the two microsatellites, Ple53 and locus Ple56 (Gaur *et al.*, 2006). From these, 28 samples amplified successfully on at least one of the microsatellites. Seven faecal samples showed signs of contamination, i.e. the samples contained genetic material from two or more lions and were not used for further analyses, leaving us with 21 samples. Both microsatellite markers showed relatively high genetic variation. Ple53 carried six alleles (among 15 individuals) and had an expected heterozygosity (assuming Hardy–Weinberg equilibrium of the encountered alleles) of 0.80. Ple56 carried seven alleles (among 14 individuals) and had an expected heterozygosity of 0.77. This high degree of genetic diversity indicates that the lions in the reserve consisted of, at least until rather recently, a large and/or open population.

The 21 samples that amplified successfully consisted of at least eleven individuals, if assuming that allelic drop-outs had affected the scored genotypes and 16 individuals, if assuming no allelic drop-outs. This number is an underestimate of the total population size as the effort curve (Fig. 2) is not levelling off, which would be expected if the number of genetically identified individuals approached the population size or if the population was highly inbred.

When the observed heterozygosity and expected heterozygosity were compared, it was found that both markers had a lower observed heterozygosity than expected at random mating ($H$ observed = 0.61 and 0.57 in Ple53 and Ple56 respectively). This may indicate that the population is partially inbred ($F_{is}$ = 0.21). Alternatively, it may be because of allelic drop-outs, which would overestimate the observed homozygosity. However, multiple polymerase chain reactions (PCR) were performed to minimize the risk of such genotyping errors. More samples and markers are needed to be run in the future to draw stronger conclusions about the potential occurrence of allelic dropouts at particular loci and the frequency of inbreeding in the population.

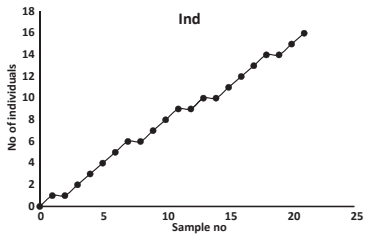


**Fig 2** Effort curve showing the cumulative number of new individual genotypes encountered in comparison with the number of successful samples

The use of noninvasive survey method through the collection of faeces is of utmost importance in conservation genetics as it allows for genetic studies of wild animals without trapping them or even observing them (Taberlet & Luikart, 1999). Additionally, noninvasive sampling techniques like in this case can increase sample sizes (Taberlet, Waits & Luikart, 1999), hence enhancing estimation of important population parameters (Parsons, 2001). Genetic sampling often provides data on the health status of a population, like inbreeding, which cannot be obtained easily using observation in the field. However, the amplification of DNA may have potential setbacks because DNA might be degraded resulting in low quantity and/or poor quality DNA available for use. This can lead to scoring errors such as false alleles (when an allele appears to have more or fewer repeats than it truly has) and 'allelic dropout' where only one allele of a heterozygous is often detected (Taberlet & Luikart, 1999), thereby producing false homozygotes leading to genotyping errors. Amplification failures during PCR and false alleles can also affect population size estimates (Taberlet, Waits & Luikart, 1999). Several methods which have been proposed to limit these genotyping errors and their subsequent impacts on analyses have been considered in this study (c.f. Taberlet, Griffin & Goossens, 1996; Schwartz, Tallmon & Luikart, 1999; Mills *et al.*, 2000; Miller, Joyce & Waits, 2002; Paetkau, 2003; Piggot *et al.*, 2004).

During the study, lions were observed by us (TT and UO) or reported by rangers on 43 different occasions including 95 different individual sightings. A more detailed analysis of the observational data, considering the sex, age and pride size and locations, suggests that a minimum of 35 individuals were observed comprising about 22 females, eight males and five juveniles. The largest pride encountered hunting or feeding was made up of six individuals (two males and four females). From the molecular data of this study, it can be concluded that there are at least 16 lions in Yankari Game Reserve.

Combining the molecular estimate of the population size (≥16 lions) and the observational estimate (minimum 35

lions), it can be concluded that within the studied area, the lion population contains at least 35 individuals. The population of lions in the remaining part of the reserve, which was not covered, is probably small or even nonexistent, although further investigations are needed to show this.

This pilot study demonstrates that analyses based on a few microsatellite loci combined with observational counts could be sufficient for an accurate estimate of the size of this (and other) lion populations. The continuous monitoring of this population will prove the strength and reliability of using faecal sample DNA to estimate population size accurately.

## Acknowledgement

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## References

Bauer, H. & Van Der Merwe, S. (2004) Inventory of free ranging lions *Panthera leo* in Africa. *Oryx* **38,** 26–31.

Bensch, S., Andrén, H., Hansson, B., Pedersen, H.-C., Sand, H., Sejberg, D., Wabakken, P., Åkesson, M. & Liberg, O. (2006) Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS ONE* **1,** e72. doi:10.1371/journal.pone.0000072.

Crick, H.Q.P. & Marshall, P.J. (1981) The birds of Yankari Game Reserve, Nigeria: their abundance and seasonal occurance. *Malimbus* **3,** 103–113.

Ernest, H.B., Penedo, M.C.T., May, B.P., Syvaren, M. & Boyce, W.M. (2000) Molecular tracking of mountain lions in the Yosemite valley region in California: genetic analysis using microsatellites and faecal DNA. *Mol. Ecol.* **9,** 433–441.

Gaur, A., Shailaja, K., Singh, A., Arunabala, V., Satyarebala, B. & Singh, L. (2006) Twenty polymorphic microsatellite markers in the Asiatic lion (*Panthera leo persica*). *Cons. Gen.* **7,** 1005–1008.

Geerling, C. (1973). *The Vegetation of Yankari Game Reserve, its Utilization and Condition*. Bulletin 3: Department of Forestry, University of Ibadan.

Green, A.A. (1989) Avifauna of Yankari Game Reserve, Nigeria: new records and observations. *Malimbus* **5,** 17–30.

Höss, M., Kohn, M., Kauner, F., Der Schröeder, W. & P'ääbo, S. (1992) Excremental analysis by PCR. *Nature* **359,** 199.

Janečka, J.E., Jackson, R., Zhang, Y., Li, D., Munkhtsog, B., Buckley_Beason, V. & Murphy, W.J. (2008) Population monitoring of Snow Leopards usin nonininvasive genetics. *Cat News* **48,** 7–10.

Kohn, M.H. & Wayne, R.K. (1997) Facts from faeces revisited. *TREE* **12,** 223–227.

Kohn, M.H., Eric, C.Y., Kamradt, D.A., Haught, G., Sauvajot, R.M. & Wayne, K.R. (1999) Estimating population size by genotyping faeces. *Proc.R Soc. Lond. B* **266,** 657–663.

Macdonald, D.W. (1980) Patterns of scent marking with urine and faeces amongst carnivore communities. *Symp. Zool. Soc. Lond.* **45,** 107–139.

Miller, S.D., Joyce, P. & Waits, L.P. (2002) Assessing allelic dropout and genotyping reliability using maximum likelihood. *Genetics*, **160,** 357–366.

Mills, L.S., Citta, J.J., Schwartz, M. & Tallmon, D. (2000) Estimating animal abundance using non-invasive DNA sampling: promise and pitfalls. *Ecol. Appl.* **10,** 283–366.

Nowell, K. & Jackson, P. (Compilers and editors) 1996. *Wild Cats: Status Survey and Conservation Action Plan. IUCN/SSC Cats Specialist Group*. IUCN, Gland, Switzerland.

Paetkau, D. (2003) An empirical exploration of data quality in DNA-based population inventories. *Mol. Ecol.* **12,** 1375–1387.

Parsons, K.M. (2001) Reliable microsatellite genotyping of dolphin DNA from faeces. *Mol. Ecol.* **1,** 341–344.

Piggot, M., Bellemain, E., Taberlet, P. & Taylor, A. (2004) A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions. *Conserv. Genet.* **5,** 417–420.

Schwartz, M.C., Tallmon, D. & Luikart, G. (1999) Using genetics to estimate the size of wild populations: many methods, much potential, uncertain utility. *Anim. Conserv.* **2,** 321–323.

Taberlet, P., Griffin, S. & Goossens, B. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Res.* **24,** 3189–3194.

Taberlet, P. & Luikart, G. (1999) Non-invasive genetic sampling and Individual Identification. *Biol. J. Linn. Soc.* **68,** 41–55.

Taberlet, P., Waits, L.P. & Luikart, G. (1999) Noninvasive genetic sampling: look before you leap. *Trends Ecol. Evol.* **14,** 323–327.

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# INDIVIDUAL IDENTIFICATION AND GENETIC VARIATION OF LIONS (*PANTHERA LEO*) FROM TWO PROTECTED AREAS IN NIGERIA

V

# INDIVIDUAL IDENTIFICATION AND GENETIC VARIATION OF LIONS (*PANTHERA LEO*) FROM TWO PROTECTED AREAS IN NIGERIA

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## ABSTRACT

This survey was conducted in two protected areas in Nigeria to genetically identify individual lions and to determine the genetic variation within and between the populations. We used faecal sample DNA, a non-invasive alternative to the risky and laborious task of taking samples directly from the animals, often preceded by catching and immobilization. Data collection in Yankari Game Reserve (YGR) spanned through a period of five years (2008 -2012), whereas data in Kainji Lake National Park (KLNP) was gathered for a period of three years (2009, 2010 and 2012). We identified a minimum of eight individuals (2 males, 3 females, 3 unknown) from YGR and a minimum of ten individuals (7 males, 3 females) from KLNP. The two populations were found to be genetically distinct as shown by the relatively high fixation index ($F_{ST}$ = 0.17) with each population exhibiting signs of inbreeding (YGR $F_{IS}$ = 0.49, KLNP $F_{IS}$ = 0.38). The genetic differentiation between the Yankari and Kainji lions is assumed to result from large spatial geographic distance and physical barriers reducing gene flow between these two remaining wild lion populations in Nigeria. To mitigate the probable inbreeding depression in the lion populations within Nigeria it might be important to transfer lions between parks or reserves or to reintroduce lions from the zoos back to the wild.

**Keywords:** faecal DNA, non-invasive sampling, protected area, isolated population, African lion

# INTRODUCTION

Human pressure, agricultural developments and industrialization are leading increasingly to the destruction, fragmentation and isolation of natural populations [1]. A consequence of these changes is loss of genetic variability [2] and increasing risk of extinction [3-5]. Their requirements for large home ranges, low fecundity and low numbers have made mammalian carnivores vulnerable to local extinction in fragmented habitats [6]. Mammals are often the dominant carnivores where they occupy the top position in the food chain, thereby serving as ecologically fundamental species for the stability of the ecosystem. Their decline or extinction may disrupt the food chains and alter the structure of ecological communities [7]. Sadly over the years, the activities of man have put on verge the future prospects of existence of many wild mammal species, especially large carnivores [8].

Lions (*Panthera leo*) once roamed most parts of Africa, Southern Europe, the Middle East and Asia [9]. Today they are only found in sub-Saharan Africa and at one locality in India where they are being increasingly restricted to protected areas and often in declining numbers [10-11]. In West Africa, lions are found only in protected areas such as national parks, game reserves and zoos. In Nigeria, the only protected areas known to still have wild lions are Yankari Game Reserve and Kainji-Lake National Park. The number of lions in these two isolates in West Africa has been poorly investigated. Population size estimate is an important biological parameter necessary for proper implementation of conservation measures [12]. Thus it is important to get adequate information on population size and connectivity between fragments of populations for proper conservation and management of a species [13]. Lions just like other large terrestrial carnivores are usually very difficult to count due to their elusive behavior and ability to cover large home ranges. An effort to conduct complete counts of a lion population is thus likely to be both organizationally difficult and time consuming [14].The alternative is to interpolate population sizes using different sampling strategies.

The use of DNA from non-invasive samples such as faeces, saliva, hairs or feathers for individual genetic tags can provide useful information for population monitoring as well as contributing with important genetic parameters [15]. Non-invasive sampling is widely used in genetic studies of elusive animal populations [16-18].This method is of prime importance in conservation genetics and behavioral ecology because it allows for genetic studies of wild animals without having to catch or even directly observe the animals under study [19], thereby reducing the possible amount of stress and harm inflicted on the animal. Various studies have employed the use of non-invasive sampling to identify individuals in a population, estimate population size [20, 12, 21, 16, 22], to monitor population sizes over time [23-24] and to also estimate the home range of individuals [20]. By studying the appropriate nuclear markers (most often microsatellites) analysis of non-invasive genetic samples (e.g. faeces) collected opportunistically from the field can provide individual identification, adequate information on population size, sex identification as well as genetic polymorphism within and between populations [25]. Knowledge of past events in a population and genetic structure is very important to assess the risk of extinction and chances of regional persistence of lion populations in Nigeria and elsewhere. Conservation of the genetic diversity of a species is important for preserving endangered wildlife [26]. This is because genetic diversity is the raw material for evolutionary change; that will allow the population to evolve in response to catastrophic changes such as new disease outbreak, pests, competition and predators.

In a pilot study carried out on the lions in Yankari Game Reserve (YGR from here on), Central North-Eastern Nigeria, between 2007-2008, we employed a population survey using genetic analysis of faecal DNA and showed the feasibility and reliability of the method [27]. In the present study, we employed the same method of faecal sample DNA analyses increasing the number of loci from two to nine to improve the precision of individual assignment. We also extended the survey to Kainji-Lake National Park (KLNP from here on), western Nigeria, which is a protected area that contains the most closely located and presumably only other Nigerian lion population to Yankari. We aimed to identify individual lions in YGR and KLNP and to determine the degree of genetic variability within and between these populations. Because the lions in the two parks have been isolated for several years, we expect them to be genetically differentiated. Also, the small population sizes suggest that there is a substantial level of inbreeding, which should be reflected by a positive inbreeding coefficient.

# MATERIAL AND METHODS

### Ethics

The study was carried out with permission from the National Park service in the case of KLNP. In YGR, the A .P. Leventis Ornithological Research Institute had a Memorandum of Understanding (MoU) with the Bauchi State government to conduct any type of ecological research. Faeces are not part of the animal and as such faecal samples are not banned by CITES from being transported between countries.

### Study sites

Faecal samples were collected from two protected areas: Yankari Game Reserve (YGR) and Kainji-Lake National Park (KLNP) (Fig. 1).

The YGR is located in Central North-Eastern Nigeria with a landmass of 2,244 km$^2$ (9° 50'N and 10° 30'E). Detail site description for YGR is contained in Tende et al. [27].

KLNP is located in the western part of Nigeria (10° 22'N 04° 33'E) and occupies a landmass of 5,340 km$^2$. The vegetation is made up primarily of Guinea savanna woodland; common woodland species include *Terminalia macroptera* found along the Oli River which flows in the centre of the Park, *Detarium microcarpum* and *Borkea africana* woodland occupy about 70% of the Park area. *Isoberlinia tomentosa* woodland play vital role in providing shelter and cover for game. The mean annual rainfall is between 1000 and 1200 mm per year and occurs between April and October, with the highest peak of rain in September [28].

### Sample collection and extraction

A total of 3724 hours were spent sampling in YGR during data collection between 2008 and 2012, and 294 hours were spent sampling in KLNP in 2009, 2010 and 2012. The Global Positioning System (GPS) was used to record the position of each sample collected. Field methods are described in detail in Tende *et al*. [27]. All samples were preserved in 95 % ethanol at room temperature, thereafter taken to Lund University, Sweden and kept in a freezer at -40°C before DNA extraction. DNA was extracted from all samples collected from YGR (n = 836) and KLNP (n = 93). DNA extraction was carried out using the QIAamp® DNA Stool Mini Kit (Qiagen).

Contamination of DNA during extraction or the PCR process can be a major problem when using non-invasive DNA; this was carefully taken care of by conforming to guidelines to avoid this through the use of a blank to control for contamination during the extraction and PCR processes.

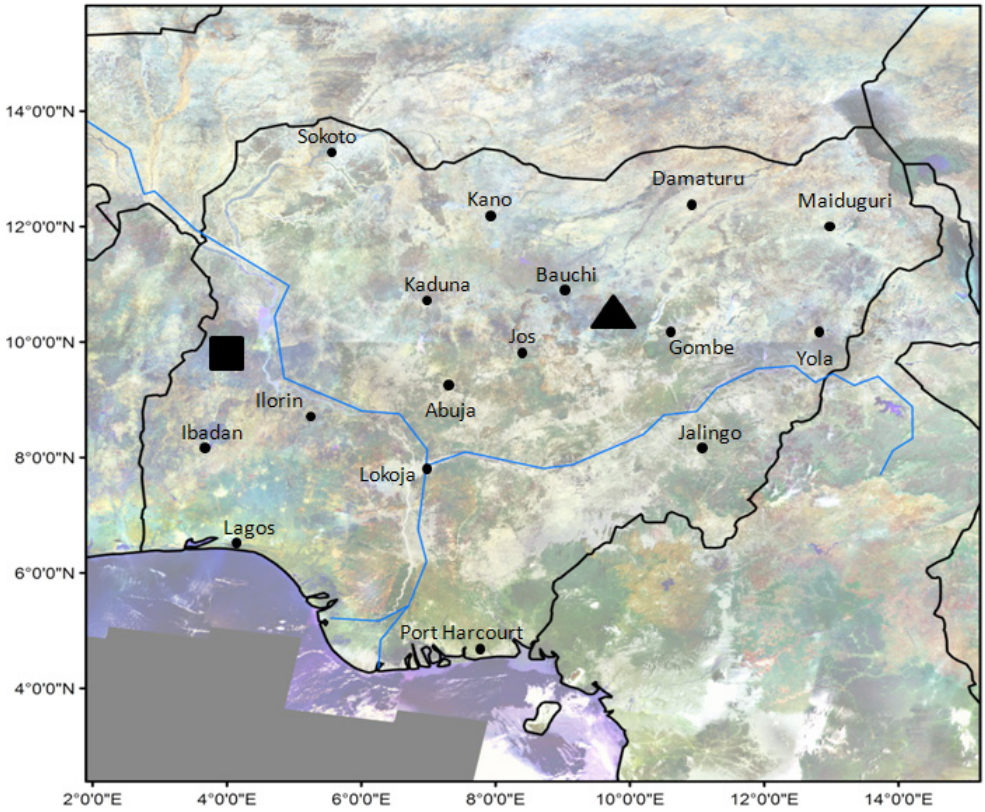


**Figure 1:** Map of Nigeria with some major cities and position of the two survey sites Kainji-Lake National Park (KLNP) in black rectangle, Yankari Game Reserve (YGR) in black triangle.

### Mitochondrial DNA

A short (206bp) portion of the mitochondrial cytochrome *b* gene was amplified and sequenced using PCR-based methods with locus specific primers to confirm samples that were from lions and to exclude a few cases of spotted hyena (*Crocuta crocuta*) or striped hyena (*Hyaena hyaena*). The primers LIHYF (5'-ATGACCAACATTCGAAAATCWC-3') and LIHYR (5'-ATGTGGGTSACTGATGAG-3') were designed to avoid amplification of human and ungulate DNA in general, in order to promote detection of the target species [27]. All amplifications were done using 2X Qiagen multiplex PCR kit in 10µl reaction volume containing 5 µl Qiagen multiplex PCR buffer mix; 0.2 µM forward primer (Applied Biosystems), 0.2 µM reverse primer, 2.6 µl water and 2 µl of DNA extract with a hot start at 95°C for 15 minutes. PCR profiles consisted of 35 cycles as follows: 90°C for 30 seconds; annealing temperature of 52°C for 30 seconds with elongation period of 72°C for 30 seconds. A blank control (reagents only) from DNA extraction process was included in all PCRs to monitor for contamination. The results of the PCR were evaluated by electrophoresis using 2% agarose gels and GelRed™ (Biotium) staining. Positive samples were sequenced using LIHY forward

primer (BigDye sequencing kit; Applied Biosystems) in an ABI Prism® 3100 capillary sequencer (Applied Biosystems).

The sequences were aligned against species reference sequences (Ascension numbers; EF437586.1, AJ809332.1 and EF107524.1) obtained from Genbank to determine species identity.

### Microsatellite amplification and genotyping

All lion samples were scored for allelic variability at nine polymorphic microsatellite primers (FCA001, FCA008, FCA026, FCA031, FCA045, FCA077, FCA126, FCA506 and FCA567 [29]. PCR amplifications were performed in 6 µl reactions containing 0.12 µl (concentration: 10 µM) dye-labelled (6-Fam, Hex or Ned) F-primer, 0.12 µl unlabelled R-primer (concentration: 10 µM), 3 µl of 2X Qiagen Master mix, 0.76 µl double distilled water and 2 µl DNA extract. PCRs were done in a GeneAmp 9700 thermocycler (Applied Biosystems) with the following profiles: 95°C for 15 min; 40 cycles at 94°C 30 s, 52°C 90 s, and 72°C 90 s; followed by an elongation period at 72°C for 10 min. Primers were multiplexed together in batches based on differences in fragment length and dye. The primer combinations are as follows: FCA001-FCA026-FCA031, FCA008-FCA045-FCA126, and FCA077-FCA506-FCA567. After amplification, alleles of the PCR products of the multiplex 3 loci, labelled with different dyes and of different lengths were separated using capillary electrophoresis in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Alleles were sized relative to GS500 ROX size standard and proof read and scored in Geneious vs. 5.6.6 (Biomatters).

### Molecular sexing

Sex of identified individuals were determined using X and Y specific primers (SMCX17 and DBY7); [30]. The primers have been designed to avoid non-target amplification [31]. PCR amplifications were performed in 6 µl reactions containing 0.12 µl F-primer (concentration: 10 µM), 0.12 µl R-primer (concentration: 10 µM), 3 µl of 2X Qiagen Master mixes, 0.76 µl double distilled water and 2 µl DNA extract). PCR profile conditions for the multiplex amplification of the SMCX17 and DBY7 fragments are as follows: An initial denaturation of 95°C for 15min, 20 cycles of 94°C for 30 sec, 60°C for 40 sec (decreasing 0.5 per cycle) and 72°C for 90 sec, followed by 20 cycles of 94°C for 30 sec, 50°C for 40 sec and 72°C for 90 sec and a final elongation period of 72°C for 15 min. After amplification, 2.5 µl of each PCR product was evaluated using 2% agarose gel with GelRed™ (Biotium) staining and samples with one band were scored as females (XX) and samples with two bands as males (XY).

### Allelic drop-out

Most non-invasive sampling studies are often confronted with low quantity and quality DNA (i.e. degraded DNA) making it ideal to use PCR primers that amplify short DNA fragments [32, 33]. Highly degraded DNA may cause allelic drop-out which results in heterozygotes being typed as homozygotes due to failure of amplification of one of the alleles. To minimize or avoid genotype errors due to allelic drop out and false alleles three independent PCRs were performed for each locus and samples as suggested by Taberlet *et al*. [34]. No alleles were retained in further analysis unless they had been detected at least twice.

Partial genotypes are assigned in some individuals at some loci where only one allele could be observed more than once. Although there is a possibility that the partial genotypes might belong to a new individual, this method of assigning them with matching samples ensure conservative population

estimation [35] by minimizing individuals created through erroneous, multi-locus genotypes (non-existent individuals). Only samples that amplified at between 4 and 9 loci were included in further analysis. According to Murphy *et al.* [36] a minimum of four loci are sufficient for accurate individual identification.

We scored samples as being from the same individual if they had been scored as the same sex, identical genotypes at ≥ four loci, and if the mismatching locus could be explained by allelic dropout. Such analyses allowed us to discern both the number of unique individuals as well as the number of "re-captured individuals". Faeces with the same multilocus genotypes are considered as recaptures.

**Genetic Analysis**

We used the identity analysis module in the program CERVUS [37] to identify individuals with unique genotypes within the data set. We also calculated number of alleles (K), allelic richness (A), observed ($H_{OBS}$) and expected ($H_{EXP}$) levels of heterozygosity, probability of observing identical genotypes by chance among unrelated samples ($P_{(ID)}$) and probability of observing identical genotypes by chance among siblings ($P_{(ID)sibs}$)from the microsatellite genotype data. The probability of identity, $P_{(ID),}$ describes the probability that two individuals which are drawn at random from a population will have the same genotypes at multiple loci [25]. The software program CREATE [38] was used to create input files for use in the software program FSTAT v2.93 [39]. Inbreeding coefficient ($F_{IS}$), population fixation index ($F_{ST}$) and Jost's estimate of genetic differentiation ($D_{est}$) were calculated using FSTAT and GenAlEx 6.5 [40]. Test for deviations from Hardy-Weinberg equilibrium exact test within populations was calculated based on 1000 randomisations, bootstrapping over loci at 95% CI. The nominal statistical significance value of 5/100 was adjusted for multiple comparisons using the Bonferroni correction to minimize possible type I error. $F_{ST}$ is used instead of $R_{ST}$ [41] because it is considered to be a more reliable estimate of genetic differentiation when using small data set with less than 20 loci.

Waypoints of sampled genotypes were laid out on site maps to provide an overview of areas they were sampled during the survey in the two study sites (Fig. 2a and b). The software package Wild1[42] in R was used to obtain Minimum Convex Polygon of all individuals and afterwards the extension Xtools (version 9.2) in ArcView 3.3 was used to calculate the area covered by each individual in order to have an overview of their movement patterns. For individuals that were encountered more than three times, their home ranges were estimated by using the minimum convex polygon method. Since at least a minimum of three points are needed to make a polygon, this was computed only for individuals that were encountered at least three times during the survey.

## RESULTS

Out of 929 samples collected from the two protected areas (YGR n = 836 and KLNP n = 93), 713 were successfully amplified for the partial cytochrome *b* (YGR, n = 625 and KLNP n = 88). The 216 samples that did not amplify at all were excluded from further analyses. Hence, the overall amplification success rate was about 77 %. This is higher than the success rate of 40 % (108 samples) that was obtained in the 2008 data from YGR [27]. There was no observed difference in the number of samples that successfully amplified in YGR and KLNP.

We determined species identity by aligning our sequences to Genbank reference sequences of the mitochondrial cytochrome *b* gene of lions, spotted hyena and striped

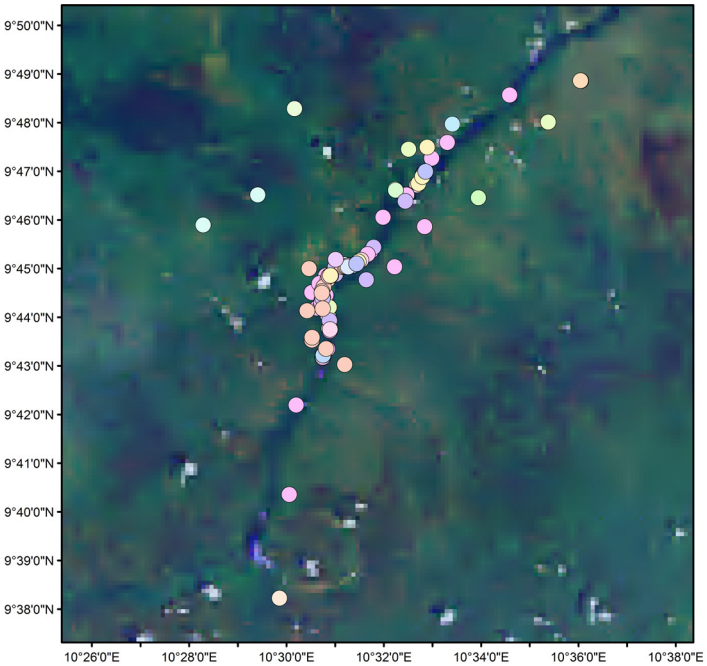


**Figure 2a:** All genotypes sampled along the core area of Yankari Game Reserve.

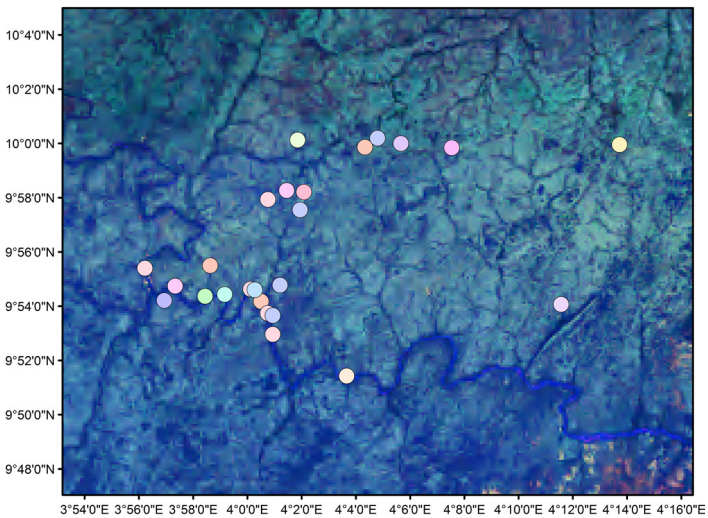


**Figure 2b**: All genotypes sampled along the core area of Kainji-Lake National Park.

hyena. Based on five informative nucleotide positions, 300 (42 %) sequences out of the 713 samples matched with spotted hyena, while the remaining samples (n = 413) were found to match with lion.

To get individual identification, the 413 samples found to contain lion DNA from the two areas (YGR n = 352 and KLNP n = 61) were genotyped at the nine polymorphic microsatellites loci.

For the nine loci we obtained complete genotypes (at all 9 loci) for 115 samples from

YGR and for 39 samples from KLNP. Partial genotypes (<9 loci) were obtained from additional 185 and 22 samples from Yankari and Kainji, respectively. Fifty two samples that amplified at less than four markers (i.e. 0 - 3 loci) were discarded from further analyses. Hence, the total number of samples with informative genotypes (≥ 4 loci) was 361.

In YGR the nine loci had a mean of 3.33 alleles with an expected heterozygosity ($H_{EXP}$) of 0.63 (assuming Hardy-Weinberg equilibrium) and an observed heterozygosity ($H_{OBS}$) of 0.32, whereas KLNP had a mean of 6.44 alleles with an expected heterozygosity ($H_{EXP}$) of 0.82 and observed heterozygosity ($H_{OBS}$) of 0.51 (Table 1). The lion populations in Kainji and Yankari showed significant signs of inbreeding with $F_{IS}$ = 0.49 in YGR and $F_{IS}$ = 0.38 in KLNP ($p < 0.001$, Table 1). The two populations were found to be genetically differentiated with $F_{ST}$ = 0.17 and $D_{est}$ = 0.65 (bootstrapping over loci the ± 95 % CI of $F_{ST}$ was between 0.10 - 0.23, p = 0.004).

A total of eight individuals (2 males, 3 females, 3 unknown) were identified in YGR if we assume that allelic drop-outs had affected the scored genotypes (Table 2), while ten individuals were identified in KLNP (7males, 3 females, Table 3). The 3 individuals in YGR whose sex could not be

**Table 1:** Summary of genetic diversity; number of alleles (K), allelic richness (A), sample size (N), Observed and Expected heterozygosity ($H_{OBS}$ & $H_{EXP}$) and inbreeding coefficient ($F_{IS}$) in the two populations over the years

| | *Yankari Game Reserve (N = 8)* | | | | | | *Kainji-Lake National Park (N=10)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | N | K | A | $H_{OBS}$ | $H_{EXP}$ | $F_{IS}$ | N | K | A | $H_{OBS}$ | $H_{EXP}$ | $F_{IS}$ |
| FCA001 | 8 | 4 | 3.61 | 0.50 | 0.60 | 0.29 | 5 | 4 | 4.00 | 0.25 | 0.82 | 0.72 |
| FCA008 | 5 | 3 | 2.77 | 0.20 | 0.51 | 0.63 | 10 | 6 | 4.06 | 0.40 | 0.77 | 0.50 |
| FCA026 | 8 | 3 | 2.88 | 0.37 | 0.62 | 0.41 | 10 | 9 | 5.66 | 0.70 | 0.90 | 0.23 |
| FCA031 | 4 | 2 | 2.00 | 0.50 | 0.65 | 0.14 | 10 | 6 | 4.71 | 0.20 | 0.83 | 0.76 |
| FCA045 | 8 | 2 | 2.00 | 0.00 | 0.53 | 1.00 | 8 | 6 | 4.49 | 0.57 | 0.79 | 0.29 |
| FCA077 | 8 | 6 | 4.37 | 0.70 | 0.80 | 0.07 | 8 | 6 | 4.61 | 0.83 | 0.87 | -0.05 |
| FCA126 | 8 | 2 | 2.00 | 0.00 | 0.53 | 1.00 | 8 | 5 | 3.80 | 0.37 | 0.71 | 0.49 |
| FCA506 | 7 | 3 | 2.44 | 0.14 | 0.69 | 0.86 | 8 | 7 | 5.05 | 0.50 | 0.85 | 0.43 |
| FCA567 | 8 | 5 | 3.93 | 0.50 | 0.72 | 0.32 | 10 | 9 | 5.43 | 0.80 | 0.87 | 0.08 |
| **Mean** | - | 3.33 | 2.88 | 0.32 | 0.63 | 0.49 | - | 6.44 | 4.64 | 0.51 | 0.82 | 0.38 |

**Table 2**: Identified individuals in Yankari Game Reserve. Shown are; Sample identity (SampleID), individual identity (IndvID), sex and allelic length at the nine scored loci, *** (indicates missing data).

| | | | | **Microsatellite Loci** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SampleID | IndvID | Sex | #times sampled | FCA001 | FCA026 | FCA031 | FCA567 | FCA007 | FCA506 | FCA008 | FCA045 | FCA126 |
| YGR321 | Y#1 | - | 4 | 129/155 | 128/128 | ***/*** | 85/85 | 135/144 | 244/244 | ***/*** | 125/125 | 124/124 |
| YGR242 | Y#2 | F | 107 | 127/127 | 128/137 | 242/244 | 94/96 | 146/153 | 214/214 | 117/117 | 127/127 | 124/124 |
| YGR211 | Y#3 | - | 5 | 129/155 | 128/128 | ***/*** | 85/85 | 130/135 | 244/244 | ***/*** | 125/125 | 124/124 |
| YGRB1 | Y#4 | M | 6 | 127/129 | 128/130 | ***/*** | 85/85 | 135/141 | 244/244 | 124/124 | 125/125 | 124/124 |
| YGRN1 | Y#5 | M | 21 | 127/127 | 128/128 | 242/242 | 94/105 | 141/153 | 191/191 | 117/117 | 127/127 | 127/127 |
| YGR13 | Y#6 | F | 14 | 127/127 | 128/137 | 244/244 | 96/105 | 141/153 | 191/214 | 117/117 | 127/127 | 127/127 |
| YGR56 | Y#7 | F | 1 | 137/155 | 130/130 | ***/*** | 85/85 | 135/135 | ***/*** | ***/*** | 125/125 | 124/124 |
| YGR7 | Y#8 | - | 1 | 127/127 | 137/137 | 242/244 | 94/107 | 153/153 | 214/214 | 117/117 | 127/127 | ***/*** |

**Table 3:** Identified individuals in Kainji-Lake National Park. Shown are: Sample identity (SampleID, individual identity (IndvID), sex and allelic length at the nine scored loci, *** (indicates missing data)

| | | | | **Microsatellite loci** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SampleID | IndvID | Sex | #times sampled | FCA001 | FCA026 | FCA031 | FCA567 | FCA077 | FCA506 | FCA008 | FCA045 | FCA126 |
| **KLNP13** | K#1 | M | 4 | 127/129 | 130/137 | 234/246 | 98/103 | 148/153 | 193/*** | 129/133 | 127/127 | ***/*** |
| **KLNP18** | K#2 | M | 1 | ***/*** | 122/130 | 224/224 | 78/78 | ***/*** | 229/229 | 129/129 | ***/*** | 129/191 |
| **KLNP19** | K#3 | M | 1 | ***/*** | 139/141 | 238/238 | 81/85 | 137/141 | 203/214 | 125/125 | 139/153 | 133/151 |
| **KLNP4** | K#4 | M | 3 | ***/*** | 134/139 | 238/252 | 83/85 | 137/139 | 195/203 | 119/133 | 139/139 | 139/139 |
| **KL26** | K#5 | M | 1 | 145/145 | 141/141 | 238/238 | 83/85 | 137/153 | 195/195 | 125/125 | 139/139 | 133/133 |
| **KL18** | K#6 | F | 7 | ***/*** | 137/137 | 246/246 | 103/105 | 148/151 | 191/191 | 133/133 | 145/149 | 131/139 |
| **KL11** | K#7 | F | 7 | 153/153 | 134/141 | 238/238 | 83/85 | 137/139 | 195/195 | 125/125 | 139/139 | 133/133 |
| **KL9** | K#8 | M | 1 | 129/129 | 128/130 | 234/234 | 98/111 | 148/153 | 85/191 | 133/133 | ***/*** | 139/139 |
| **KL33** | K#9 | F | 1 | 153/153 | 134/139 | ***/*** | 85/85 | 153/153 | 193/208 | 117/133 | ***/*** | 133/133 |
| **K22** | K#10 | M | 1 | 145/153 | 144/148 | 244/244 | 83/94 | 137/*** | 197/203 | 127/129 | 149/153 | 133/*** |

determined molecularly were due to shortage of DNA template. Based on the observed allele frequencies there was a low probability of observing identical genotypes $P_{(ID)}$ from two randomly sampled individuals from the same population in both YGR ( $P_{(ID)}$ =0.00000213, $P_{(ID)sibs}$ = 0.00259) and KLNP( $P_{(ID)}$=0.00000000150, $P_{(ID)sibs}$ = 0.000188). The mean polymorphic information content (PIC) values in both YGR (0.50) and KLNP (0.73) were high.

Within both survey areas, some of the identified individual genotypes were encountered in more than one year (YGR; n= 6, KLNP; n=3), while some were encountered several times within a year and others were encountered only once or twice (figs 3a and 3b). In YGR individual Y#2 (female) and Y#5 (male) were observed to be present in the population all through the five year period. Individual Y#6 (female) was sampled from the beginning of the survey until 2011 (Fig. 3a). The combined genotypes of Y#2 and Y#5 are compatible with the hypothesis that the genotypes of Y#6 and Y#8 are their offspring (Table 2).

In both YGR and KLNP all individuals were sampled within the core area of the reserve (Fig. 2a and b). The home range analysis using the minimum convex polygon method gave an average home range of 11.91 $km^2$, ± 2.1 SD (1.71– 47.62 $km^2$) in YGR (Appendix 1) and 26.75 $km^2$ ± 1 SD (14.63–39.37 $km^2$) in KLNP (Appendix 2).

# DISCUSSION

### Number of individuals

Based on the encounter of unique genotypes during the different survey years, a minimum of eight individuals were identified in YGR between 2008 and 2012, while ten individuals were identified in KLNP between 2009, 2010 and 2012. During the course of the survey there has been at least one observation of new cubs in the field in 2010 in YGR. Recently, there have also been sightings (between 8th and 12th March 2013) of 2 adult females with 3 cubs, 2 adult females without any cubs, 1 adult male with a mane together with 3 cubs in YGR. The sightings of these adults within YGR (1 male and 4 females, or 1 male and 2 females, (if we assume that it was the same females that were seen at different times) contradict the findings of Wildlife Conservation Society (WCS) who has reported, through call-up stations, that only two adult lions exist within the reserve [43]. These sightings are also confirmed by

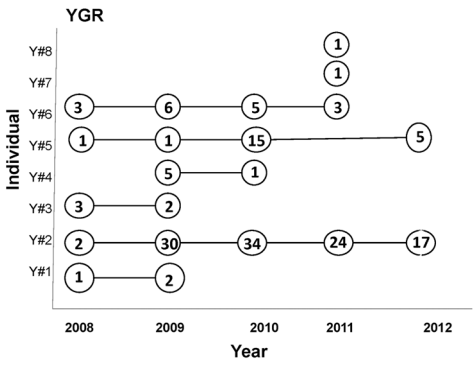


**Figure 3a**: Sampling frequency per individual and year in Yankari Game Reserve.

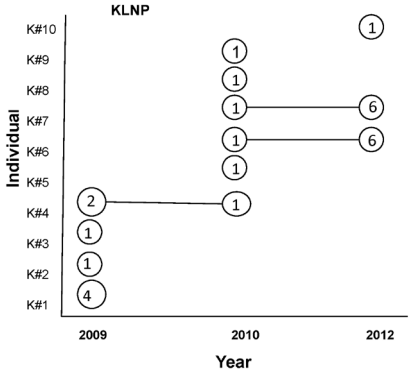


**Figure 3b**: Sampling frequency per individual and year in Kainji-Lake National Park.

our molecular findings (Table 2), where females Y#2 and Y#6 and male Y#5 have been present most parts of the survey periods. Six of the eight identified individuals have been sampled multiple times (3-107 times) during the survey (Fig 3a). Our estimate gives the minimum number of lions that exist in both YGR and KLNP. Since several individuals have been encountered only once, especially in KLNP, the true number is potentially higher. The low estimates of probability of identity ($P_{(ID)}$ and $P_{(ID)sibs}$) obtained in our study both in YGR and KLNP gives confidence to the identity of individuals identified in these two survey areas and support the uniqueness of the identified individuals. Waits *et al.* [44] suggested that these values should be between $P_{(ID)}$ = 0.01 – 0.0001 in studies estimating population size.

Despite the fact that there were fewer visits and also few samples collected from KLNP, we observed more individuals as well as a higher genetic diversity than in YGR. This suggests that there are and have been more individuals in the recent history in KLNP, which have contributed to a larger effective population size and a higher genetic diversity. Alternatively, the high diversity in KLNP may have resulted from connecting with gene flow with lions in the W-Arli-Pendjari complex, a vast protected area in Benin, Burkina Faso and Niger.

**Comparison with previous study**

A number of studies conducted on the natural populations of African lions have been carried out mostly in Eastern, Central and Southern Africa and in India. These studies have employed opportunistic field observation and/or genetic data to understand behavioral, social and breeding structure of the lions [45-53]. Researchers have incorporated molecular methods to understand the genetic status of the lions in their natural populations [c.f. 50, 54], characterize their evolutionary history, as well as to study disease outbreaks [55- 58]. Our study has employed non-invasive samples with aid of molecular techniques to make available information on the population size as well as the genetic status of the remaining relict wild lion populations in Nigeria. The method does not have any negative impact on the study species.

The number of individuals estimated in YGR in the present study is lower than the number recorded during the pilot survey conducted by Tende *et al.* [27] where eleven individuals were identified from two microsatellite loci. This difference could be due to either a disappearance from the population of some individuals due to natural deaths or to the activities of poachers within the reserve, or it could be an overestimation in our previous study due to the low number of loci used. The use of polyacrylamide gel to genotype individuals during our pilot study [27] could possibly also have biased our estimation due to bad gels where stutter bands might have been typed as true bands.

During the course of the laboratory analysis and efforts to optimize different primers for the study, we ran short of DNA template from most of the extracted samples from the first pilot study in YGR. Hence, not all samples analyzed in our 2008/2009 pilot survey could be rerun on the new microsatellite primers used. However, in some samples where we still had DNA template it was observed that certain individuals have persisted in the population throughout the survey years.

The higher amplification success rate attained in this study as compared to 2007/2008 could be attributed to the new PCR kit/ technique (Qiagen multiplex PCR kit) that was employed and also we concentrated our sample efforts on fairly fresh faeces. The shorter time the faeces are left out in the environment, the less degradation of DNA and this is especially true in tropical environments

where high temperature and UV radiation, can cause fast degradation of DNA [59].

**Home range estimates**

Many of the identified individual genotypes were encountered several times during the study. Lions are known to use large home ranges to satisfy energetic demands, but this can be limited where required resources have clumped distribution [50, 60]. This is the case during the dry season, for both study sites, when game concentrates close to rivers - Gaji River in YGR and Oli River in KLNP. Both rivers run through the core areas of the respective reserve/park and offer lush vegetation for prey species, as well as cover for the lions to rest and also stalk. It is also the main source of drinking water both for the predators and the prey. A similar survey by Spong *et al.* [50] to determine space use by lions in Selous Game Reserve, Tanzania, showed the most intensively used area to be small within the reserve and averaged only 11.7 km$^2$, which is consistent with our finding in YGR where individuals were sampled mostly within the core areas which are intensively used and this averaged at 11.9 km$^2$ (SD: ± 2.1). They found out that prides often had close relatives in neighbouring prides but in areas with high prey abundance the home ranges between individuals, irrespective of relatedness tended to overlap more [50]. A survey by Lehmann *et al.* [60] on home range utilization of lions in Karongwe Game Reserve, South Africa, showed the home range used by a pride ranged between (10.3 - 64.4 km$^2$) and for a single male ranged between (5.0 - 56.3 km$^2$). Their findings about home range size are slightly larger than what we have obtained in our study (YGR: 1.71- 47.62 km$^2$; KLNP: 14.63-39.37 km$^2$). In our study a female (Y#2) was observed to have the largest home range (47.52 km$^2$), this might probably be due to the need to hunt and feed the cubs and the male. This female has also been observed to persist in the population since the onset of this survey in 2007/2008. Both of the above studies [50, 60] employed the use of telemetry. Our study has shown that genotype data also can be used to determine the home range of lions, and possibly other mammals, enhancing sample size and reducing disturbance to the animal under study.

**Inbreeding**

The populations of lions in both KLNP and YGR exhibit significant signs of inbreeding. This is not surprising given their small population sizes. The inbreeding level found in YGR in the present study is in line with our pilot study conducted in 2008 [27] when the inbreeding coefficient was estimated to be 0.21, whereas in this present survey the value was found to be 0.49 (Table 1). The inbreeding levels in both YGR (0.49) and KLNP (0.38) are high and comparable to what has been recorded in some other carnivore species. For instance the estimated inbreeding coefficient in the Scandinavian wolf (*Canis lupus*) population was up to 0.41 [61, 62, 17] before the population was genetically rescued by one immigrant from Finland [63]. The arrival of this immigrant into the Scandinavian wolf population provided the possibility to avoid inbreeding, decrease the risk of inbreeding depression and cause population growth. High inbreeding coefficient reaching up to 0.37 has also been recorded in the brown bear (*Ursus arctos*) [64]. Inbreeding and subsequent negative effects of inbreeding have been reported in the lions in Ngorongoro Crater in Tanzania [65, 48]. The number of alleles at a set of microsatellite loci for the Etosha lion population (A = 4.6) reported by Lyke et al. [53], and that reported by Antunes et al. [58] (A = 4.4) is similar to that detected in KLNP (A = 4.6), but higher than in YGR (A = 2.8), where Lyke et al. [53] detected no sign of inbreeding in the

Etosha population ($F_{IS}$=0.03). The inbreeding coefficient recorded in our study is higher than what has been recorded in the lion population in Etosha National Park in Namibia. Some studies [e.g. 4, 66 - 68] have shown that if populations remain small and isolated for many generations they are bound to face increased inbreeding and gradual erosion of genetic variability. The lion populations in Nigeria are small, isolated and restricted to two protected areas (YGR and KLNP) where their populations may be declining. This is of course a threat to the long-term survival of these populations and to the lion population throughout West Africa [69].

Low genetic variability has been reported in lions in Ngorongoro Crater in Tanzania [48, 55, 70, 71]. Although there are recent observations of cubs within one of our study systems (YGR) the genetic status of this population as shown by our study is poor. This might affect individual survival and reproductive success in the long term if proper measures are not implemented. O'Brien [72] has observed that there is a strong correlation between genetic variation and reproductive parameters in lions. This means that with time the fitness of individuals within the lion populations in Kainji and Yankari will decline due to accumulation and expression of recessive and detrimental alleles with subsequent inbreeding depression, which might consequently drive the population to extinction.

**Population structure**

The YGR and KLNP populations were found to be genetically differentiated ($F_{ST}$ = 0.17, $D_{est}$ = 0.65). This is not surprising due to the fact that these populations are small and isolated from each other; about 1000 km apart and they are separated by dispersal barriers including highways, agricultural landscapes and cities. Without any corridor for dispersal, isolation is expected to build up the observed pattern of allelic differentiation between the two populations. Moreover, high human and livestock densities characterize most of the surroundings of these protected areas, which can increase mortality risk of cubs and "possible dispersers" because of overlap with human habitations and livestock. All these factors may act as barriers to gene flow between the two populations and possible populations in neighboring countries. Studies have shown that geographical and environmental features can influence gene flow and genetic variation within and between populations [73, 74]. Studies carried out in California on mountain lion (*Puma concolor*), and coyote (*Canis latrans*) and bobcat (*Lynx rufus*) to assess the level of gene flow and differentiation in these carnivores showed how anthropogenic obstacles such as roads, agriculture and urbanization were great barriers to dispersal and gene flow [75, 76]. It was shown how these barriers imposed artificial home range boundaries on territorial carnivores thereby decreasing genetically effective migration, which leads to population differentiation.

A study carried out by Antunes *et al*. [58] to assess genetic variation from 357 lions from most of its current range in Africa and Asia using microsatellite data revealed significant population structure ($F_{ST}$ = 0.03 - 0.79) within East-African lion populations despite the low geographic distance within them. Their finding is in accordance with our finding about the two lion populations in Nigeria ($F_{ST}$=0.17).

The existence and maintenance of genetic diversity and good connectivity between subpopulations are essential factors for long-term viability of a population [77], which should serve as the primary target of any acceptable conservation management program. Genetic diversity is the raw material needed in order to evolve the ability to cope with environmental challenges such as disease outbreak and parasites [78].

**Conservation genetics and management**

The two lion populations are small and genetically different with signs of inbreeding within each population. This might elevate their risk of extinction in the face of sudden environmental catastrophes in the future. It has been shown that small populations are often faced with a higher risk of extinction from environmental catastrophes [3, 4, 5, 79-83] than large interconnected populations [84, 77, 85].

The main aim of a conservation genetic approach is to maintain diversity within and between populations so as to enhance the evolutionary potential of the population to cope with environmental changes. Laboratory and translocation experiments have indicated that small and inbred populations can be rescued by contribution of minimal number of immigrants [86-88, 62, 63]. This can help to decrease inbreeding and inbreeding depression [86, 89], and bring about profound changes in genetic structures [90, 91, 63]. Thus to mitigate the inbreeding condition and subsequent probable inbreeding depression in the lion population within Nigeria it might be important to transfer lions between parks or reserves or to reintroduce lions from the zoo back to the wild. This is necessary in order to enhance their genetic diversity for long term survival and reproduction. The bringing together of genetically dissimilar mates, hybrid vigor [89-91] can be advantageous because it will enhance reproductive success and also fitness [92-93 68]. However, it would be recommended that lions that are genetically similar to the receiving population should be preferably used in any translocation program in order to avoid introduction of potentially non-locally adapted genotypes.

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## REFERENCES

1. Ceballos G, Ehrlich PR (2002) Mammalian population losses and the extinction crisis. Science 296: (5569) 904 - 907.
2. Frankham R (1995) Inbreeding and Extinction- a Threshold Effect. Conserv Biol 9 (4): 792-799.
3. Saccheri I, Kuussaari M, Kankare M, Vikman P, Fortelius W, Hanski I (1998) Inbreeding and extinction in a butterfly metapopulation. Nature 392: 491- 494.
4. Bijlsma R, Bundgaard J, Boerema AC (2000) Does inbreeding affect the extinction risk of small populations?: predictions from Drosophila. J Evol Biol 13: 502-514.
5. Higgins K, Lynch M (2001) Metapopulation extinction caused by mutation accumulation. Proc Natl Acad Sci USA 98: 2928-2933.
6. Crooks KR (2002) Relative sensitivities of mammalian carnivores to habitat fragmentation. Conserv Biol 16: 488-502.
7. Crooks KR, Soule ME (1999) Mesopredator release and avifaunal extinctions in a fragmented system. Nature 400: 563-566
8. Morris DW, Kotler BP, Brown JS, Sundararaj V, Ale SB (2009) Behavioral indicators for

conserving mammal diversity. Ann. New York. Acad. Sci. 1162: 334-356

9.Bauer H, Vander Merwe S (2004) Inventory of free ranging lions *Panthera leo* in Africa. Oryx 38: 26-31.

10. Nowell K, Jackson P (Compilers and editors) (1996) Wild cats: Status survey and Conservation Action Plan. IUCN/ SSC Cats Specialist Group. IUCN, Gland, Switzerland.

11. Wilson DE, Mittermeier RA. eds (2009) Handbook of the Mammals of the World. Vol.1. Carnivores. Lynx Ediciones. Barcelona.

12. Kohn MH, York EC, Kamradt DA, Haught G,Sauvajot RM, Wayne RK (1999) Estimating population size by genotyping faeces. Proc R Soc Lond B 266: 1-7.

13. Creel S, Spong G, Sands JL, Rotella J, Zeigle J, Joe L, Murphy KM, Smith D (2003) Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. Mol Ecol 12: 2003-2009.

14. Balme GA, Hunter LTB, Slotow R (2007) Evaluating Methods for Counting Cryptic Carnivores. J Wildl Mgt 73 (3): 433-441.

15. Bellemain E, Taberlet P (2004) Improved non-invasive genotyping method: application to brown bear (*Ursus arctos*) faeces. Mol Ecol Notes 4: 519-522.

16. Ernest HB, Penedo MCT, May BP, Syvaren M, Boyce WM (2000) Molecular tracking of mountain lions in the Yosemite Valley region in California: genetic analysis using microsatellites and faecal DNA. Mol Ecol 9: 433-441.

17. Bensch S, Andren H, Hansson B, Pedersen H-C, Sand H, Sejberg D, Wabakken P, Åkesson M, Liberg O (2006) Selection for heterozygosity gives hope to a wild population of inbred wolves. *PLoS ONE* 1(1): e72. doi:10.1371/journal.pone.0000072.

18. Zhang H-M, Guo Y, Li D-S, Wang P-Y, Fang S-G (2009) Sixteen novel microsatellite loci developed for the giant panda (*Ailuropoda melanoleuca*). Conserv Genet 10: 589-592.

19. Taberlet P, Luikart G (1999) Non-invasive genetic sampling and Individual Identification. Biol J Lin Soc 68: 41-55.

20. Taberlet P, Camarra J-J, Griffin S et al. (1997) Nonivasive genetic tracking of the endangered Pyrenean brown bear population. Mol Ecol 6: 869-876

21. Woods JG, Paetkau D, Lewis D, McLellan BN, Proctor M, Strobeck C (1999) Genetic tagging free-ranging black and brown bears. Wildl Soc Bull 27: 616-627.

22. Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Mol Ecol 10: 249-256.

23. Kendall KC, Metzgar LG, Patterson DA, Steele BM (1992) Power of sign surveys to monitor population trends. Ecol Appl 2: 422-430.

24. Schwartz M, Tallmon D, Luikart G (1998) Review of DNA-based census and effective population size estimators. Anil Conserv 1: 293-299.

25. Kohn MH, York EC, Karmradt DA, Haught G, Sauvajot RM, Wayne RK (1999) Estimating population size by genotyping faeces. Proc R Soc Lond B 266: 657-663

26. Uphyrkina O, O'Brien SJ (2003) Applying molecular genetic tools to the conservation and action plan for the critically endangered Far Eastern leopard (*Panthera pardus orientalis*).Comptes Rendus Biologies 326: 93-97(supplement).

27. Tende T, Ottosson U, Hansson B, Åkesson M, Bensch S (2010) Population size of lions in Yankari Game Reserve as revealed by faecal DNA sampling. J Afr Ecol 59: 7-12.

28. Afolayan TA (1978) The effect of fires on the vegetation in Kainji lake National Park, Nigeria. Oikos 33: 376-382.

29. Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK, O' Brien SJ (1999) A genetic linkage map of microsatellites in the domestic cat (Felis catus). Genomics 57: 9-23.

30. Hellborg L, Ellegren H (2004) Low levels of nucleotide diversity in mammalian Y chromosomes. Mol Biol Evol 21:158-163.

31. Seddon JM (2005) Canid-specific primers for molecular sexing using tissue or non-invasive samples. Conserv Genet 6: 147-149.

32. Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14(8): 323-327.

33. Pompanon F, Bonin A, Taberlet P (2005) Genotyping errors: causes, consequences and solutions. Nature 6: 847-859.

34. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable Genotyping of Samples With Very Low DNA Quantities Using PCR: Nucleic Acids Res 24: 3189-3194.

35. Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJ, Burke T (2003) Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. Mol Ecol 12: 1649-1661.

36. Murphy MA, Waits LP, Kendall KC, Wasser SK, Higbee JA, Bogden R, (2002) An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conserv Genet 3: 435-440

37. Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7: 639-655.

38. Coombs JA, Letcher BH, Nislov KH (2008) CREATE: a software to create input files from diploid genotypic data for 52 genetic software programs. Mol Ecol Res 8: 578-580 .Vs.1.31

39. Goudet J (2002) FSTAT, a computer program to estimate and test gene diversities and fixation indices Version 2.9.3.2.

40. Peakall, R, Smouse, PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288-295

41. Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. Gen Mol Biol 139: 457-462.

42. Sargeant GA (2011) wild1: R tools for wildlife research and management. R package version 1.09. U.S. Geological Survey Northern Prairie Wildlife Research Center, Jamestown, ND, USA.

43. Nyanganji G, Saidu Y, Henschel P, Dunn A (2012) 2011 survey of lion (Panthera leo) in Yankari Game Reserve and Kainji Lake National Park, Nigeria (A submitted report National Park service).

44. Waits LP, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Mol Ecol 10: 249-256.

45. Packer C, Pusey AE (1982) Cooperation and competition within coalitions of male lions: kin selection or game theory? Nature 296:740-74.

46. Pusey AE, Packer C (1987) The evolution of sex-biased dispersal in lions. Behaviour 101: 275-310.

47. Gilbert DA, Packer C, Pusey AE, Stephens JC, O'Brien SJ (1991) Analytical DNA fingerprinting in lions: parentage, genetic diversity, and kinship. J. Heredity 82: 378-386.

48. Packer C, Pusey AE, Rowley H, Gilbert DA, Martenson J, O'Brien SJ (1991) Case study of a population bottleneck: Lions of the Ngorongoro Crater Conserv Biol 5 (2): 219-230.

49. Spong G, Creel S (2001) Deriving dispersal distances from genetic data. Proc. R. Soc. Biol. Sci. Ser. B 268: 2571-2574.

50. Spong G, Stone J, Creel S, Björklund M (2002) Genetic structure of lions (*Panthera leo L.*) in the Selous Game Reserve: implications for the evolution of sociality. J Evol Biol 15: 945-953.

51. Kays RW, Patterson BD (2002) Mane variation in African lions and its social correlates. Can. J. Zool. 80: 471-478

52. Dubach J, Patterson BD, Briggs M B et al. (2005) Molecular genetic variation across the southern and eastern geographic ranges of the African lion, Panthera leo. Cons Genetics 6:15–24.

53. Lyke MM, Dubach J, Briggs MB (2013) A molecular analysis of the African lion (*Panthera leo*) mating structure and extra-group paternity in Etosha national Park. Mol Ecol DOI:10.1111/mec.12279.

54. Shankaranarayanan P, Banerjee M, Kacker RK, Aggarwal RK, Singh L (1997) Genetic variation in Asiatic lions and Indian tigers. Electrophoresis 18:1693-1700.

55. Brown EW, Yuhki N, Packer C, O'Brien SJ (1994) A lion lentivirus related to feline immunodeficiency virus: epidemiologic and phylogenetic aspects. J. Virol 68:5953-5968.

56. Roelke-Parker ME, Munson L, Packer C, Kock R, Cleaveland S, et al.(1996) A canine distemper virus epidemic in Serengeti lions

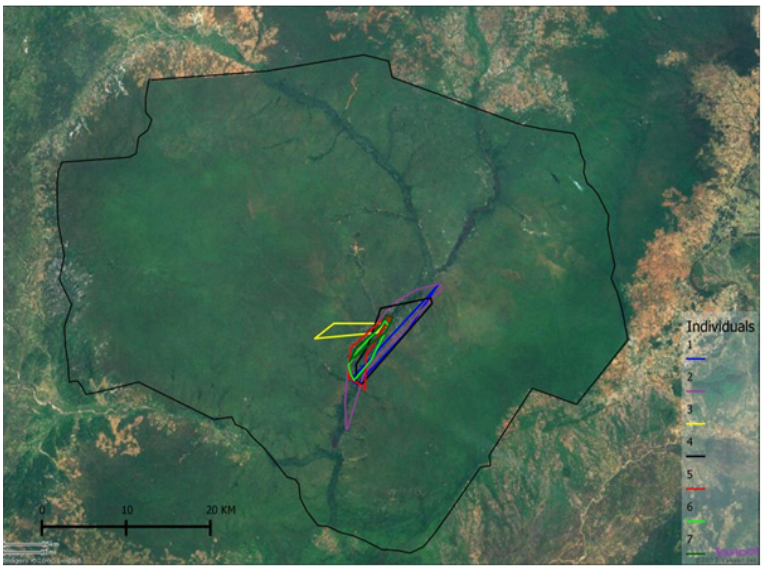
(Panthera leo).Nature 379:441-445.

57. Packer C, Altizer S, Appel M, Brown E, Martenson J, et al. (1999) Viruses of the Serengeti: patterns of infection and mortality in African lions. J. Anim Ecol 68:1161-1178.
58. Antunes A, Troyer JL, Roelke ME, Pecon-Slattery J, Packer C, et al. (2008) The Evolutionary Dynamics of the Lion *Panthera leo* Revealed by Host and Viral Population Genomics.Plos Gen 4 (11) e1000251.
59. Piggott MP, Bellemain E, Taberlet P, Taylor AC (2004) A multiplex pre-amplification method that significantly improves microsatellite amplification and error rates for faecal DNA in limiting conditions. Conserv Genet 5: 417-420.
60. Lehmann MB, Funston PJ, Owen CR, Slotow R (2008) Home Range Utilization and Territorial Behaviour of Lions (*Panthera leo*) on Karongwe Game Reserve, South Africa. PLOS ONE3(12):e3998.doi:10.1371/journal.pone.0003998.
61. Laikre L, Ryman N (1991) Inbreeding depression in a captive Wolf (Canis lupus) population. Conserv Biol 5:33-40
62. Liberg O, Andren H, Pedersen CH, Sand H, Sejberg D, Wabakken P, Åkesson M, Bensch S (2005) Severe inbreeding depression in a wolf *Cani lupus* population. Biol Lett 1:17-20
63. Vila C, Sundqvist AK, Flagstad Ø, Seddon J, Blörnerfeldt S, Kojola I, Casulli A, Sand H, Wabakken P, Ellegren H (2003) Rescue of a severely bottlenecked wolf (*Canis lupus*) population by a single immigrant. Proc R Soc Lond B. 270: 91-97.
64. Laikre L, Andren R, Lars H, Ryman N (1996) Inbreeding depression in Brown Bear (Ursus arctos). Biol Conserv 76**:**69-72.
65. Obrien SJ, Martenson JS, Packer C, Herbst L, de Vos V *et al*. (1987) Biochemical genetic variation in a geographic isolates of African and Asiatic lions. Natl Geo Res 3:114-124.
66. Lande R (1995) Mutation and conservation. Conserv Biol 9: 782-791.
67. Laikre L (1999) Conservation genetics of Nordic carnivores: lessons from zoos. Hereditas 130 (3): 203 -216.
68. Keller, L.F, Waller, D.M (2002). Inbreeding effects in wild populations. Trends Ecol Evol 17: 230-241.
69. Frankham R, Ballou JD, Briscoe DA (2002) Introduction to Conservation Genetics. Cambridge University Press Cambridge UK.
70. Munso L, Brown JL, Packer C, Janssen D, Reiziss SM, Wildt DE (1996) Genetic diversity affects morphology in free-ranging lions (Panthera leo) of the Serengeti Plains and Ngorongoro Crater. J Rep Fer 108:11-15.
71. Wildt DE, Bush M, Goodrowe KL, Packer C, Pusey AE et al. (1987) Reproductive and genetic consequences of founding isolated lion populations. Nature 329:328-331.
72. O'Brien S (1994) A role for molecular genetics in biological conservation. Proc Natl Acad Sci USA 91: 5748-5755.
73. Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. Trends Ecol Evol 18: 189-197.
74. McRae BH, Beier P, Dewald LE, Huynh LY, Keim P (2005) Habitat barriers limit gene flow and illuminate historical events in a wide-ranging carnivore, the American puma. Mol Ecol 14:1967 -1977.
75. Ernest HB, Boyce WM, Bleich VC, May B, Stiver SJ, Torres SG (2003) Genetic structure of mountain lion (*Puma concolor*) populations in California. Conserv Genet 4: 353-366.
76. Riley SPD, Pollinger JP, Sauvajot RM, York EC, Bromley C, Fuller TK, Wayne RK (2006) A southern California freeway is a physical and social barrier to gene flow in carnivores. Mol Ecol 15: 1733-1741.
77. Frankham R (1996) Relationship of genetic variation to population size in wildlife. Conserv Biol 10:1500-1508.
78. Frankham R, Kingslover JG (2004) Response to environmental change: Adaptation or extinction. In: Ferrier R., Dieckman U., Couvet D (Eds.) Evolutionary Conservation Biology. Cambridge University Press, Cambridge pp.85-100.
79. Frankel OH (1970) Variation, the essence of life. Proc Linn Soc New South Wales 95:158-169.
80. Frankel OH (1974) Genetic conservation: our evolutionary responsibility. Genetics 78:53-65.
81. Lande R (1988) Genetics and demography in biological conservation. Science 214:1455-

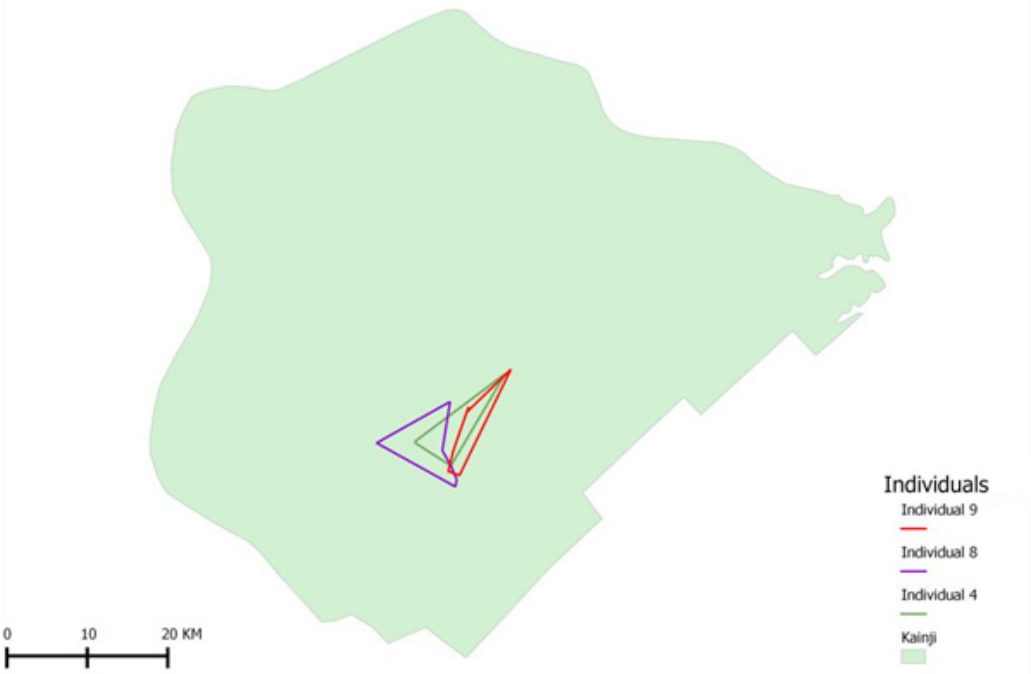
1460.

82. Woodroffe R., Ginsberg JR (1998) Edge effects and extinction of populations inside protected areas. Science 280: 2126-2128.
83. Dunham J, Peacock M, Tracy CR, Nielsen J, G. Vinyard G (1999) Assessing extinction risk: integrating genetic information. Conserv Ecol [online] **3**(1): 2. http://www.consecol.org/vol3/iss1/art2/ .
84. Lande R (1993) Risks of population extinction from demographic and environmental stochasticity and random catastrophes. Am Nat 142:911-927.
85. Lacy RC (2000) Considering threats to the viability of small populations using individual-based models. Ecol Bull 48: 39-52.
86. Spielman D, Frankham R (1992) Modelling problems in conservation genetics using captive Drosophila populations: improvement of reproductive fitness due to immigration of one individual into small partially inbred populations. Zoo Biol 11:343-351.
87. Westemeier RL, Brawn JD, Simpson SA, Esker TL, Jansen RW, Walk JW, Kershner EL, Bouzat JL, Paige KN (1998) Tracking the long-term decline and recovery of an isolated population. Science 282:1695-1698.
88. Madsen T, Shine R, Olsson M, Wittzell H (1999) Conservation biology: restoration of an inbred adder population. Nature 402: 34-35.
89. Ebert D, Haag C, Kirkpatrick M, Riek M, Hottinger JW, Pajunen VI (2002) A selective advantage to immigrant genes in a Daphnia meta population. Science 295: 485-488.
90. Ball SJ, Adams M, Possingham HP, Keller MA (2000) The genetic contribution of single male immigrants to small, inbred populations: a laboratory study using Drosophila melanogaster. Heredity 84: 677-684.
91. Saccheri IJ, Brakefield PM (2002) Rapid spread of immigrant genomes into inbred populations. Proc R Soc Lond B 269:1073-1078.
92. Amos W, WorthingtonWilmer J, Fullard K, Burg TM, Croxall JP, Bloch D, Coulson T (2001) The influence of parental relatedness on reproductive success. Proc R Soc Lond B 268:2021-2027.
93. Hedrick PW, Kalinowski ST (2000) Inbreeding depression in conservation biology. Annual Rev Ecol Syst 31:139-162.

# SUPPLEMENTARY MATERIALS




**Appendix 1**: Estimated home ranges for some individuals in Yankari Game Reserve.




**Appendix 2**: Estimated home ranges for some individuals in Kainji-Lake National Park