University of Chester

An assessment of the genetic diversity of the founders of the European captive population of Asiatic lion (*Panthera leo persica*) using microsatellite markers and studbook analysis

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Master of Philosophy by Kirsty Elizabeth Atkinson.

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The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of the thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit.

Signed…… Dated ……...

An assessment of the genetic diversity of the founders of the European captive population of Asiatic lion (*Panthera leo persica*) using microsatellite markers and studbook analysis

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Abstract

The European Endangered Species Programme (EEP) population of Asiatic lions (*Panthera leo persica*) was founded in the early 1990's from nine individuals sourced from an Indian captive population. During 2007-2009, 57 lions were born into this captive population. Of these births, 35 individuals died within 20 days, three within two months, and one individual was medically euthanized at four months. Indeed, over 50% of total historic captive population died within 30 days of birth. These stillbirths, and high levels of infant mortality, could be due to high levels of inbreeding. Previous research has recorded genetic variation in the current Indian captive population. This research uses the same microsatellite markers to establish the level of genetic variation which was captured in the establishment of the EEP population in relation to the variation observed in Indian zoo populations. At 12 markers showing variation in the Indian captive population, only two showed bi-allelic heterozygosity in the EEP founders, suggesting that variation was not captured during the establishment of the EEP population. This lack of variation was confirmed through sequencing of two mitochondrial DNA segments; cytochrome *b* and D-Loop. The 'European Studbook for the Asiatic Lion' provides some historic pedigree information showing that the EEP founder population contains offspring resulting from full-sibling and half-sibling matings, resulting in a number of inbred individuals, including all the female founders. A number of unsuitable matings have also been recorded during the last decade. Given the observed limited genetic variation at the markers tested, this study recommends the import of Asiatic lions from India (captive or wild-caught), incorporating genetic testing and studbook analysis, in order to introduce genetic variation into the EEP.

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List of abbreviations used

BSC	Biological Species Concept
CE	Capillary Electrophoresis
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
ddH2O	Double distilled water
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EEP	European Endangered Species Programme
ESU	Evolutionarily Significant Units
F	F-value (level of inbreeding)
GD	Gene Diversity
HVR	Hyper-variable region
HWE	Hardy-Weinberg Equilibrium
H_E	Expected heterozygosity
H_o	Observed heterozygosity
H_2O	Water
IUCN	International Union for Conservation of Nature

MK	Mean Kinship
MSI	Mate Suitability Index
mtDNA	Mitochondrial DNA
MU	Management Unit
$\boldsymbol{N_e}$	Effective population size
PCR	Polymerase Chain Reaction
SNP	Single nucleotide polymorphism
SSP	Species Survival Plan

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1. Introduction

1.1 Conservation Genetics

Over the last three decades our ability to assess the differences between individuals, populations and species at a genomic level has become possible through both advancements in technology and improved scientific knowledge. Whilst morphological traits can be used to confidently identify specimens at the species level of taxonomy, sub-species level can sometimes be less obvious. The use of genetic tools allows a more robust method (for example, using phylogenetics) to determine the evolutionary relationships between, and within, species and populations (Davis, 1996, Wu, 2001). This application of molecular biology to the question of evolution and speciation is termed 'Conservation Genetics'. The subject also encompasses the use of genetic tools to study differences between populations at a genetic level and allows comparisons between these populations, which may help to identify specific groups within a species which may require urgent or specific conservation efforts in order to sustain or preserve the integrity of the population. Conservation genetics can also guide the effective capture of founders when establishing captive populations, ensuring they provide a full representation of variability required (Russello and Amato, 2004, Gilbert *et al*., 1991, Goncalves da Silva *et al*., 2010, Ryder, 1986, Frankham *et al*., 2010). This variation is paramount to the long-term viability of a captive population, and is not necessarily linked to morphological traits. That is, variation may not be visible to an assessment team, or, conversely, any specific morphological variation observed may not be linked to viable genetic variation.

1.1.1 Speciation

A major aspect of conservation genetics relates to the definition of a species. Carl Linnaeus (1707-1778) established a taxonomic species identification system through the implementation of a binomial classification for vegetables, minerals and animals. This classification system is based on morphological traits, and allows each biological entity to be classified through kingdom, phylum, class, order, family, genus and finally, species. Animal (*Animalia*) classifications were originally published in the 10th edition of *Systema Naturae* (Linnaeus, 1758) and this system is still used today. Despite this, there remains an ongoing debate over the question of speciation, the concepts behind the process, and the taxonomic levels which have the most importance (Davis, 1996, Mallet, 2001, Mallet, 2007), with many authors either avoiding providing a definition or stating its impossibility to define (Frankham *et al.,* 2010, Fa *et al.*, 2011, Mallet, 2007, Wu, 2001). A common concept used to define a species is the Biological Species Concept (BSC, Mayr, 1963), which denotes speciation based on the reproductive isolation of gene flow across the entire genome. The BSC encompasses a range of mechanisms through which this isolation can occur, including allopatry (geographical separation), population bottlenecks (sudden and drastic reduction in population size) and sexual selection causing a change in phenotype (Turelli *et al.,* 2001). Conservation genetics applies the genic view of speciation, which suggests speciation occurs through differentiation leading to adaptation at the gene level (Wu, 2001, Frankham *et al.*,2010).

1.1.2 The subspecies question

Sub-species are geographically isolated populations which have been separated from a common ancestor for a large period of time but remain phenotypically similar (Mayr, 1969), encompassing both the time and space effect of speciation (Mallet, 2007). Frankham *et al.* (2010) defines a sub-species as a population displaying partial genetic differentiation from the main species, or populations which are showing divergence away from the original

species toward a new species. Identification of sub-species can then allow classification according to the vulnerability to extinction for that particular population. For example, *Panthera tigris* (tiger) is classed as endangered by the IUCN (Goodrich *et al.*, 2015) but *Panthera tigris sumatrae* (Sumatran tiger) - a sub-species - is classed as critically endangered (Linkie *et al.*, 2008).

Sub-species are still physically capable of inter-breeding, and can normally produce viable offspring. However, as the populations are normally geographically isolated, with restricted gene flow, the populations do not normally meet and depending on the length of time the populations have been isolated, breeding between sub-species may not be recommended (Wu, 2001, Frankham *et al.*, 2010).

However, there can be issues caused by both over-zealously splitting species into multiple sub-species unnecessarily, and by not defining clear and distinct sub species where necessary. The former can cause a distorted view of the apparent numbers of individuals within the sub-species and may suggest the sub-species is requiring conservation efforts, when, in fact, the translocation of animals from another sub-species or population is a plausible and acceptable solution. For example, in the early 1990's the number of Florida panthers (*Felis concolor coryi*) dropped to less than 50 as a result of hunting and fitness issues (Hedrick, 1995). Genetic research established little divergence across the sub-species range of the parent species, Puma (*Felis concolor*) (Hedrick, 1995, Seal, 1994). The research recommended the translocation of eight female Texan cougars (*Felis concolor stanleyana*) to supplement the Florida population (Seal, 1994, Packer, 2010). This translocation was deemed to be a success, with the Texas-Florida hybrids displaying improved genetic fitness and an increase in population size (Packer, 2010). Compared to capture and captivity, this translocation was less costly, potentially more effective, and offered an improved quality of life to the individuals in the population (Packer, 2010).

Conversely, grouping distinct sub-species into one species group when considering conservation efforts can cause the loss of unique genetic diversity, the eradication of entire species, or hybrids formation through cross-breeding. A documented example of this involves the now extinct dusky seaside sparrow (*Ammodramus maritimus nigrescens*), a sub-species of seaside sparrow (*Ammodramus maritimus*). Numbers of dusky seaside sparrows dropped to just six male birds in 1980; five of these individuals were caught and entered into a captive breeding programme with female Scott's seaside sparrow (*Ammodramus maritimus peninsulae*) (Avise and Nelson, 1989). The back-crossing of first generation hybrid females with dusky males was successful in producing Scott/dusky hybrids with 50-87.5% dusky genes, however, the last pure dusky seaside sparrow died in 1987, and the sub-species was declared extinct. It has since been established that the sub-species of seaside sparrow has two distinct evolutionary histories, and can be split into two groups (clades) named the 'Atlantic Coast' and 'Gulf'. The dusky seaside sparrow originated from the Atlantic Coast clade and the Scott's seaside sparrow is part of the Gulf clade. It is estimated the two clades diverged between 250,000 and 500,000 years ago, suggesting that another Atlantic Coast sub-species may have provided a more suitable option for the breeding programme.

1.1.3 Evolutionary Significant Units

The desire to define populations (within species or sub-species) which require conservation management has led to the concept of Evolutionarily Significant Units (ESU) (Ryder, 1986, Frankham *et al.*, 2010, Mallet, 2007). These units describe a population within a species or sub-species which has a unique conservation demand, or populations which contain genetic variation which may be lost if the population is not protected (Ryder, 1986). The original designations of ESU were based on reproductive isolation and unique adaptations (Ryder, 1986, Frankham *et al*., 2010) however, more recently, they tend to be designated based on

genetic differentiation from other populations of the same species (Moritz, 1994, Vogler and Desalle, 1994, Frankham *et al.*, 2010).

However, there are critics to the use of molecular tools for the purpose of defining ESU, as it is believed that the designations defined by ecological histories and adaptive traits have more relevance to the management of populations (Crandall *et al,* 2000). Crandall *et al.* (2000) defined a 'cross-hair' system to define management units (MU) based on ecological or genetic exchangeability between populations, both recent and historical, which attempts to show adaptive differentiation and the presence or absence of gene flow. Ecological exchangeability is the ability of individuals to occupy the same ecological niche, community or environment; genetic exchangeability is the assessment of the gene flow between the populations and any restrictions to that gene flow (Crandall *et al.*, 2000, Frankham *et al*., 2010).

1.1.4 IUCN classifications

Species, subspecies and/or ESU are classified by the IUCN (International Union for Conservation of Nature and Natural Resources) according to the vulnerability status, based on data gathered regarding population trends, habitat area and threat of extinction (Frankham *et al.*, 2010, Fa *et al,.* 2011, Mace and Lande, 1991). These classifications are extinct, extinct in the wild, critically endangered, endangered, vulnerable, near threatened, and least concern; some species are not classified through insufficient data or not evaluated against the criteria (IUCN, 2017, Mace and Lande, 1991). The vulnerable category is defined as having a 10% probability of extinction within 100 years (Mace and Lande, 1991, Frankham *et al.*, 2010, IUCN, 2017) and it has been suggested that this level of extinction risk is the highest level which is biologically acceptable (Mace and Lande, 1991, Shaffer, 1981).

Following a threatened classification (critically endangered, endangered or vulnerable), conservation efforts must be considered in order to protect the species and biological diversity. Decisions are made whether to apply the conservation efforts *in-situ,* such as to protect the natural habitat, provide physical protection from predators and hunting; or to remove some or all individuals from a population and place them into captivity (*ex-situ*), normally a zoo or wildlife sanctuary (Frankham *et al*., 2010, Fa *et al.*, 2011). Whilst *in-situ* conservation would generally be preferred rather than removing the species from its natural habitat, it is recognised that this is not always possible, or may not necessarily be sufficient in order to ensure the long-term protection or survival of the species and *ex-situ* conservation becomes essential (Fa *et al.*, 2011). This is particularly pertinent if habitat loss is the issue for the species in question.

Conservation efforts *in-situ* will be restricted by available finances, competing priorities in habitat ownership, the availability of scientific data, and issues surrounding local or National laws (Fa *et al*., 2011, Frankham *et al*., 2010, Frankham *et al*., 2014, Flather *et al.*, 2011). Opinions of the local human population of the species in question may hinder conservation plans, for example, in rural Kenya, elephants are seen as pests which can trample and ruin a whole year's crop in a single night, and as such, the locals would not welcome *in-situ* conservation schemes for this species (Fa *et al.,* 2011). Likewise, modern cosmopolitan zoos and wildlife parks have little interest in *ex-situ* management of species which may not be considered aesthetically pleasing to their paying visitors, regardless of their IUCN status (Fa *et al.*, 2011).

1.2 Captive Populations

Captive populations (*ex-situ* conservation) have been established for many species, because of dramatic changes in climate and ecological niches brought about mainly by human activity, in order to ensure that the species survive. Without this intervention, certain

species would have become extinct in the wild, as they would have been incapable of surviving in their natural habitat as a result of these changes (Frankham *et al*., 2010). Indeed, a number of plant and animal species only survive within captive populations, having become extinct in the wild, for example, Przewalski's horse (*Equus przewalskii*) and the Franklin tree (*Franklinia alatamaha*) (Frankham *et al*., 2010, O'Brien *et al*., 1985). The preservation of species within captivity can also be used as assistance to wild populations, by supplementing struggling populations, or maintaining the population in captivity until a suitable alternative habitat can be created or located. The main aims of captive breeding are to assist conservation by securing the populations, educating the public, allowing research to be carried out on species, and reintroduction (Ballou *et al.,* 2010, Fa *et al*., 2011, Frankham *et al*., 2010).

The effective establishment of captive populations of endangered species relies on the balance of many ecological, biological and financial factors (Fa *et al*., 2011, Barnett *et al*., 2006a, Barnett *et al*., 2006b, Dubach *et al*., 2005, Russello and Amato, 2004, Goldstein *et al*., 2000, Ryder, 1986). Fa *et al.* (2011) coined the term 'Zoo Conservation Biology' and defines the four main disciplines which contribute to this field as, captive animal management, small population biology, conservation education and translocation biology (Table 1). It is the integration and collaborative operations of all these inter-disciplinary fields which will benefit captive populations and long-term conservation efforts.

1.2.1 Founder populations

During the pre-establishment of a captive population, the sub disciplines of 'small population biology' and 'translocation biology' (Table 1, Fa *et al.,* 2011) are the key drivers regarding which, and how many, individuals will be required to ensure capture of genetic diversity relative to the diversity found in the wild population. Specialist knowledge should be employed to consider species-specific issues, such as, harem mating (where a dominant

male has multiple partners and sires all the offspring in the group), age of sexual maturity, life span, mating system, litter size, fecundity and gestation period (Dubach *et al*., 2005, Ryder, 1986). This knowledge and experience should be applied to advise the optimal founder numbers, and target individuals, for each particular species (Frankham *et al.*, 2010, Fa *et al*., 2011, Shafer *et al.,* 2015, Frankham *et al.*, 2014).

Table 1. Disciplines and sub-disciplines contributing to Zoo Conservation Biology. Table adapted from Fa *et al.,* (2011).

	Zoo Conservation Biology discipline			
	Captive Animal Management	**Small Population Biology**	**Conservation Education**	**Translocation Biology**
Sub-discipline examples	Nutrition Reproduction Environment Psychological well-being Disease prevention	Inbreeding Demography Genetics Estimate of extinction Minimum viable population	Education Theory Formal learning Informal learning Visitor motivation studies Learning principles	Ecology Costs Effectiveness Project Management Re-introducability of species

Individuals are either captured from the wild, or donated from other previously founded captive populations (Frankham *et al.,* 2010). Only individuals who have contributed genetically (i.e. have reproduced within the captive population) are classed as founder individuals when assessing the population (Ballou *et al*., 2010). The financial resources required to establish and maintain captive populations make it essential that these founder individuals will be the most likely to generate a viable, long-term population (Goldstein *et*

al., 2000, Goncalves da Silva *et al*., 2010, Ivy *et al*., 2009). It is vital that these founder individuals possess as much of the genetic diversity found within the wild population as possible (Goldstein *et al*., 2000, Goncalves da Silva *et al*., 2010, Ivy *et al*., 2009, Frankham *et al*., 2010).

1.2.2 Effective population size

In order to determine the rate of inbreeding and loss of genetic variation, the population census number (N) is not commonly used as an indicator, given that not all members of the population will be involved in breeding within the population. Effective population size (N_e) is a number which expresses the genetically effective numbers within the population and is usually lower than the number of breeding individuals (Frankham *et al*., 2010, Ballou *et al*., 2010). As the N_e allows for number of breeders at age of sexual maturity, unequal sex ratios, non-random mating and overlapping generations, it provides a better estimation of how a particular population is expected to maintain genetic diversity over time than census numbers (Ballou *et al*., 2010 , Frankham *et al*., 2010). Unequal sex ratio, family size variation and fluctuation in population size were deemed by Frankham (1995a) to be the key factors which influence the accuracy of estimates of N_e.

Provided as a ratio of N_e/N allows a direct comparison between species, and long-term effective population sizes are often around one-tenth of the census size (Frankham *et al*., 2010). In a study assessing N_e/N estimates from 102 species, a mean N_e/N ratio of 0.11 was estimated with adjustments made for unequal sex ratio, family size variation and fluctuation in population size, and a mean N_e/N ratio of 0.34 was estimated with no adjustments made (Frankham, 1995a). Given that the former figure would be most representative of the true ratio in a physical population (rather than theoretical), this ratio falls below the minimum ratio of 0.2 assumed in the Mace-Lande categorisations of endangered species (Mace and Lande, 1991).

1.2.3 Minimum viable populations

Given the demand for space within captivity, and the ever-increasing numbers of species requiring captive breeding programmes, the minimum number of individuals required to maintain the population and the reproductive fitness of the individuals is an essential consideration (Frankham *et al.*, 2010, Fa *et al.,* 2011, Lacy, 2013). An inherent issue within captive populations is that inbreeding will increase over every generation in any finite, captive population, unless new founders are introduced, regardless of the original founder numbers used (Blouin, 2003, Frankham *et al*., 2010, Basset *et al*., 2001). The inbreeding rate increases at $1/(2N_e)$ per generation (Frankham *et al*., 2010, Soulé, 1980).

There have been attempts to define a universal MVP number for all species to meet the two main goals of captivity; one short term (avoid inbreeding depression) and the second long term (maintain evolutionary potential) (Frankham *et al*., 2014, Frankham *et al*., 2010, Franklin, 1980, Flather *et al.*, 2011, Brook *et al.*, 2011, Soulé, 1980, Lande and Barrowclough, 1987). It is essential to understand that MVP numbers must be the equivalent N_e number, not the census number.

The thresholds of N_e of 50 to avoid inbreeding depression and N_e of 500 to retain evolutionary potential were initially proposed in 1980 (Soulé, 1980, Franklin, 1980) and became known as the 50/500 rule. The N_e of 50 to avoid inbreeding depression is derived from the $1/(2N_e)$ equation to allow an inbreeding rate of 1% per generation (Soulé, 1980, Frankham *et al*., 2014). The N_e of 500 to retain evolutionary potential was derived from the balance between loss of genetic diversity through genetic drift and the added variation achieved during genetic mutations (Frankham *et al*., 2014, Traill *et al*., 2010).

However, criticisms have since been levied against the 50/500 rule, both for its generic cross-species nature and for the low values of these numbers (Brook *et al.*, 2011, Flather *et*

al., 2011, Frankham *et al.*, 2014). The cross-species general rule is defended by the oftentimes need to take urgent action in matters of protective conservation, normally in situations where data on the species in question are sparse, and there are a lack of numbers available for study in the wild (Frankham *et al.,* 2014, Flather *et al*., 2011). It has recently been suggested that the numbers should be increased to 100/1000 (Frankham *et al.,* 2014) and others have proposed the latter number should be in the region of 5000 (Traill *et al*., 2010). However, a critique of conservation management practice suggests that the regardless of the numbers required for evolutionary potential to be maintained, and advice on the matter from experts involved in evidence-based research, the policies and targets of conservation organisations do not encompass these figures (Traill *et al.*, 2010). Ironically, the species requiring conservation efforts often have very small population sizes and it is impossible to meet the MVP criteria, even if the finances and socio-political opinions were in concord with the MVP requirements (Traill *et al.*, 2010, Frankham *et al.*, 2010).

1.2.4 Captive Breeding Programmes

The consideration of evolutionary significance of zoo populations is an essential aspect of any captive breeding programme, especially those involved in programmes where reintroduction is a key aim, where populations within captivity must remain exchangeable (ecologically and/or genetically) with their wild counterparts (Goldstein *et al*., 2000, Goncalves da Silva *et al*., 2010, Ballou *et al.,* 2010, Lacy, 2013, Schulte-Hostedde and Mastromonaco, 2015, Fa *et al*., 2011). The guiding principle and common goal of captive populations is to retain at least 90% of the source variation for at least 100 years (Goncalves da Silva *et al*., 2010, Ivy *et al*., 2009, Russello and Amato, 2004, Lacy, 2013, Frankham *et al*., 2010, Frankham *et al.*, 2014, Ballou *et al.,* 2010).

The capture of large numbers of individuals from the wild may be cost-prohibitive; however, long-term consideration must be given to the long-term survival and viability of the population. Where few individuals are used to establish the captive population, the cost to maintain this population will be increased in order to maintain genetic diversity; where large numbers of founders are captured from multiple locations, the cost of maintaining the population long-term is decreased (Frankham *et al*., 2010).

There have been successful instances of captive breeding programmes, one of which is the black-footed ferret (*Mustela nigripes),* a carnivore member of the weasel family. After numbers declined throughout the 20th century through the extermination of their main prey source, the prairie dog (*Cynomys* spp*.),* the last known remnant population of black-footed ferrets was heavily affected by canine distemper and plague in the mid-1980's (Belant *et al*., 2015, Forrest *et al.,* 1988). The species was declared extinct in the wild by 1996 and is still classified as endangered (Belant *et al*., 2015, Wisely *et al.,* 2008). The captive breeding programme was initiated in 1987, where the decision was made to capture all the remaining known black-footed ferrets from the wild; 18 individuals were captured, which was believed to be the entire wild population at that time (Russell *et al.*, 1994). Since 1991, over 3900 black-footed ferrets have been released back into the wild at 24 reintroduction sites, resulting in nearly 300 breeding adults in the wild populations in the 2015 census (Belant *et al*., 2015). All wild-living individuals which exist today all descend from, or are, captive-bred individuals (Belant *et al*., 2015).

Whilst the successful reintroduction of a self-sustaining wild population should, rightly, be applauded, success stories are often hard to find. In a review of 145 captive breeding programmes (with reintroduction as an aim) during the 1990's, there were only 16 cases which had undergone a successful reintroduction process (Snyder *et al*., 1996). Often the captive populations are not self-sustaining, and ironically, require import of animals from

the wild, rather than the captive population supplementing the wild population (Fa *et al*., 2011).

Captive breeding programmes initiated with good intent often face other challenges, even when all the key disciplines (Table 1) and MVP are considered prior to the establishment of the initial founder population. Critiques of captive breeding programmes suggest that they should not been seen as a long-term solution, and should only be implemented when all other options have been exhausted (Frankham, 2008, Snyder *et al*., 1996). One group of authors have suggested a wild population with acutely low numbers may have a better chance of long-term survival by being left in the wild, than to be put into captivity and risk the deleterious issues that may ensue by this action (Snyder *et al*., 1996). The same authors were critical of the concept, and management, of many zoological institutions suggesting they are driven by the financial benefits of the species they exhibit, rather than the longevity of the species survival in the wild (Snyder *et al*., 1996). They did, however, by express their understanding of the paradox of the issue with regards to conservation funding from external sources being sparse and generally unavailable (Snyder *et al*., 1996).

It is of concern that captive populations regularly require supplementation from the wild population rather than other way round (Fa *et al*., 2011). This practice is generally carried out to reduce the effects of captivity on a species, particularly the genetic effects, however, in endangered species, this further supplementation may either not be possible or further endanger any conservation efforts for the wild population (Fa *et al.*, 2011). One specific example of this occurring is through the import of nearly 600 wild African elephants (*Elephas maximus*) to supplement captive populations around the world following research declaring the non-self-sustaining nature of the captive population (Fa *et al.*, 2011, Wiese, 2000).

One of the most common issues within closed captive populations is the risk of inbreeding . It is commonly accepted that inbreeding is an accepted consequence of the nature of these closed, captive populations (Frankham *et al.*, 2010, Fa *et al.*, 2011). However, often the rate of inbreeding exceeds the accepted rate due to unexpected breeding behaviours and/or unknown and unqualified relatedness of founders (Frankham *et al.*, 2010, Fa *et al.*, 2011). Inbreeding occurs at a faster rate when closely related individuals mate as this decreases heterozygosity and can increase the occurrence of deleterious and/or recessive alleles (Fa *et al.*, 2011). Inbreeding depression is the resulting loss of fitness within these offspring and is often correlated with high infant or juvenile mortality (Fa *et al*., 2011).

Another major challenge facing any captive population is limiting genetic adaptation to captivity (Frankham, 2008, Schulte-Hostedde and Mastromonaco, 2015, Pelletier *et al.*, 2009), where behavioural or physical adaptations are observed as a response to the captivity. To reduce the occurrence of this adaptation, it is recommended to limit the number of generations a population is kept in captivity (Frankham, 2008), however, there is empirical evidence that individuals can be affected after just one generation in captivity (Christie *et al.*, 2012). Specifically, this research found that first generation hatchery-reared steelhead trout (*Oncorhynchus mykiss*) had increased survival rates, but reduced reproductive success in the wild (Christie *et al.*, 2012). This study, whilst keen to point out that it may not be the same across all species or taxa, seems to reinforce the strongly held assumption that over time, fitness traits for survival in the wild reduce rapidly within captivity (Snyder *et al.*, 1996, Frankham, 2008, Fa *et al.,* 2011). One explanation for this phenomenon is stated to be due to the increase of potentially deleterious alleles which are under selective pressure in the wild (Snyder *et al*., 1996, Frankham, 2008).

Some criticisms are directed at captive breeding programmes as a whole, based on the belief that evolution of a species is linear concept, with extinction being the final, natural

part of the timeline (Traill *et al*., 2010, Frankham *et al*., 2010). Species have been going extinct since life began on earth, although stated extinction rates vary greatly (normally based on computer modelling) (Frankham *et al*., 2010, Shaffer, 1981), although over 1000 plant and animal species have been recorded extinct in the last 400 years (Smith *et al.,* 1993). It is clear that extinctions will continue, without question. Therefore, the criticisms levied at captive breeding programmes are often related to their purpose, and, by taking animals into captivity, whether we are interfering in the natural evolution, and extinction, of species (Snyder *et al*., 1996). Whilst this appears to be a valid point, we, as humans, have to take some responsibility of the effects our everyday practices have on other species. It seems that if these practices are causing a premature demise of the species in question, action should be taken in an attempt to protect these animals.

1.3 Molecular tools

Genetic data can be used for many conservation research purposes, such as, to identify species, to suggest population of origin, to ascertain pedigree of population, to match a sample source to an individual and to allow an assessment of genetic variation (Alacs *et al*., 2010, Ogden *et al.*, 2009). Whilst the choice of molecular marker will depend on the desired analysis level there are multiple genetic methods which have been developed over the last 30 years, alongside the technology to support these. These range from hybridisation techniques (restriction fragment length polymorphisms (RFLP)) to amplification techniques, which relied on the development of the polymerase chain reaction (PCR) by Kary Mullis in the early 1980's (Mullis *et al.*, 1986). PCR is a molecular technique which artificially replicates DNA over multiple temperature cycles, exponentially replicating the segment(s) of target DNA. This key technique has revolutionised the field of DNA analysis, and has led to the ability to rapidly increasing the amount of target DNA for downstream applications, such as gel visualisation or DNA sequencing. Whilst there is some variation in mutation rates, with significant differences observed across different animal groups, different loci

and even different lineages within species, there are recognised 'universal' estimates for different markers (both nuclear and mitochondrial), allowing a prediction of molecular divergence over time (Avise, 1994).

1.3.1 Mitochondrial DNA markers

DNA barcoding is one genetic technique which uses sequence divergence in mitochondrial DNA (mtDNA) to define and identify species (Hebert *et al.,* 2003). Universal sites on the mitochondrial genome are used, such as, cytochrome *b* and cytochrome oxidase 1, which have conserved flanking regions, meaning prior knowledge of the species is not required (Hebert *et al.,* 2003, Alacs *et al*., 2010, Ogden *et al.*, 2009). Given the relative abundance of mtDNA in each cell, degraded and/or trace level samples can be amplified more consistently than nuclear DNA (Frankham *et al*., 2010, Alacs *et al*., 2010, Ogden *et al.*, 2009). The resulting DNA fragments can be sequenced, and it is the differences in these sequences that allows the species identification. That is, these regions are conserved within a species (intra-species) but differ between species (inter-species) (Alacs *et al.*, 2010). Research has stated that the mutation rate of mtDNA is around 5-10 times faster than that of nuclear DNA (Butler, 2005, Brown *et al.*, 1979, Nachman and Crowell, 2000). However, the hypervariable region of the mtDNA accounts for much of this increased rate, and the mutation rates for the coding areas (encompassing the cytochrome *b* and cytochrome oxidase 1 genes) are similar to the rate of speciation (Ogden *et al.*, 2009, Avise and Nelson, 1989), which aligns the marker with its use as a species identification tool. The more closely related the species, the more similar the DNA sequences are within these areas. These specific areas of mtDNA are often used as a phylogenetic tool to infer relatedness or divergence points of different species in evolutionary history (Frankham *et al.*, 2010).

1.3.2 Nuclear DNA markers

For population genetics, and individual genotyping, nuclear DNA is most commonly used, specifically microsatellite markers, or short tandem repeats (STR). These areas (loci, singular- locus) are found across the genome, and consist of a pattern of repeated nucleotide bases, where the number of repeats differ between individuals. The mutation rate for these areas of the nuclear genome are the highest within the genomic DNA as they are generally found in non-coding regions (Butler, 2005, Gymrek, 2017), which reiterates their importance within population genetics and individual identification.

By separating out these polymorphisms by size, it is possible to designate a genotype profile at each loci tested (Butler, 2005). By testing multiple polymorphic loci, it is possible to discriminate at an individual level. However, these markers tend to be species specific and require much research to develop and characterise the levels of polymorphism found.

Prior to the establishment of any captive population, genetic analysis should be carried out in order to resolve any taxonomic issues, identify specific populations or management units causing concern and detecting hybridisation with other species (Frankham *et al*., 2010). By carrying out this analysis it guides the capture of effective founders both in terms of numbers and the right source of individuals in a genetic context. However, in the case of the establishment of the captive population of Asiatic lion, amongst others, predates the availability of the analysis of genetic data, and as such, the population was established without access to, or consideration of this information (O'Brien *et al*., 1985, Boakes *et al*., 2007).

1.4 *Panthera leo*

Panthera leo (Linnaeus, 1758) is the taxonomic name for lions (Kingdom: Animalia; Phylum: Chordata; Class: Mammalia; Order: Carnivora; Family: Felidae), classified as 'Vulnerable' by the IUCN (Bauer *et al.,* 2016). Historically, *P. leo* was found extensively across Africa,

Europe, the Middle East and Asia up to the mid to late Pleistocene (Antunes *et al*., 2008, Barnett *et al*., 2006b, Bauer and van der Merwe, 2004, Dubach *et al*., 2005, Patterson, 2007, Barnett *et al*., 2009, Barnett *et al*., 2006a, Burger *et al*., 2004, O'Brien *et al*., 1987a, Driscoll *et al*., 2002, Shankaranarayanan *et al*., 1997). During the late Pleistocene mega-faunal crash, the lion's range was severely reduced, and the species disappeared from Europe around 2,000 years ago due to the growth of dense forest (Antunes *et al*., 2008, Barnett *et al*., 2006b, Bauer and van der Merwe, 2004, Dubach *et al*., 2005, Mazak, 2010, Barnett *et al*., 2009, Bertola *et al*., 2011, Haas *et al*., 2005). This restriction caused a separation of two main sub-species, the African Lion population and a remnant population of lions which were geographically isolated in the Gir Forest refuge in India (Figure 1), forming the Asiatic lion sub-species (*P. l. persica*) (Bauer and van der Merwe, 2004, Dubach *et al*., 2005, Shankaranarayanan *et al*., 1997, Barnett *et al*., 2006a, O'Brien *et al*., 1987a, Driscoll *et al*., 2002, Singh and Gibson, 2011, Wildt *et al*., 1987, Haas *et al*., 2005).

Figure 1. Image of location of Gir National Park, India. Inset shows close up of region 'A' highlighted on the map of India. (Image sourced from maps.google.co.uk).

This affirmation of a sub-species occurred through allozyme genotyping done by a group of researchers who tested lions from three distinct African populations (Serengeti, Ngorogoro Crater and Kruger Park) and Gir Forest Asiatic lions (O'Brien *et al*., 1987b). Seven of the 46-50 allozyme loci tested were polymorphic in the African Lions only; all 46 allozyme loci tested in the Gir Forest Asiatic lions were monomorphic (O'Brien *et al*., 1987b).

The habitat was further restricted around the turn of the 19th Century, where the lion was no longer observed in the Middle East and Northern Africa, creating further geographical distance between the two sub-species (Antunes *et al*., 2008, Barnett *et al*., 2006b, Bauer and van der Merwe, 2004, Dubach *et al*., 2005, Mazak, 2010, Shankaranarayanan *et al*., 1997, Bertola *et al*., 2011, O'Brien *et al*., 1987a). This isolation results in a greater risk of inbreeding, as gene flow is restricted, and breeding can only occur between individuals within the same population (Bertola *et al*., 2011).

Recently, questions have been raised regarding the current classification of *P. leo* encompassing all African lions (Bertola *et al.*, 2015, Dubach *et al.*, 2013, Bertola *et al.*, 2011, Antunes *et al.*, 2008), with the IUCN declaring the West African subpopulation of *P. leo* as 'Critically Endangered' in 2015 (Henschel *et al.,* 2015). The subpopulation has suffered a 66% decline in population numbers from 1993-2014 (Bauer *et al.,* 2016).

This new classification is a result of a wealth of genetic studies which have shown that the populations located in West/Central Africa are geographically and genetically distinct from the lions located in East/Southern Africa (Bertola *et al.*, 2016, Bertola *et al.*, 2015, Dubach *et al.*, 2013, Bertola *et al.*, 2011, Antunes *et al.*, 2008, Dubach *et al.*, 2005, Bauer and van der Merwe, 2004). Studies have shown greater genetic variation in the Eastern/Southern populations along with a greater numbers of lions (Bertola *et al.*, 2015, Bertola *et al.*, 2011, Dubach *et al.,* 2013, Antunes, *et al.,* 2008). This geographical divide has been observed in

other African mammals, and this has been suggested to be as a result of refuge areas during the late Pleistocene era (Bauer, 2016).

1.4.1 Panthera leo persica

Panthera leo persica is classed as 'Endangered' by the IUCN Red List (Breitenmoser *et al.*, 2008). The status of the sub-species improved in 2008 from the 2000 classification as 'Critically Endangered'. However, the reclassification is purely based on population size from the 2008 census; no other reclassification criteria were met. The population size has been increasing in numbers year on year, with 523 individuals being recorded in the 2015 census (Wildlife Conservation Trust, 2017), although it is suggested that the population has reached the expansion limits of the range of the Gir Forest (Breitenmoser *et al*., 2008). The status of *P. l. persica* has led to its inclusion on CITES (Convention on International Trade in Endangered Species) Appendix I, which prohibits International Commercial Trade in species which are threatened with extinction. Special authorisation can be given in exceptional circumstances, such as where samples may assist in medical research (CITES, 2013).

In wild populations, defence mechanisms exist for reducing the risk of inbreeding, such as, the adolescent males moving away from the pride at sexual maturity, and females accessing nomadic males as breeding partners, rather than pride males (Rudnai, 1973, Lehmann *et al.*, 2008). However, once the population becomes isolated and nomadic behaviour is limited, these activities may not be possible due to the small population size (Wildt *et al*., 1987).

There is molecular evidence for at least two population bottlenecks which have occurred in the history of *P. l. persica*. The first of these occurred around 2600 years ago, where diversity was severely and extensively homogenised by the geographic isolation of the Gir Forest population (Driscoll *et al*., 2002). This event was genetically severe, and occurred

during the spread of forested areas in Europe and the Middle East, confining an isolated population in North-Western India. This 'founder effect' reduced variability to a critical level, and it has been cited that lack of variation within the Asiatic lion is a characteristic of this bottleneck, rather than through inbreeding within the population (O'Brien *et al*., 1987b).

The population was further affected as a consequence of human predation and encroachment around the turn of the 20th century (Barnett *et al*., 2009, O'Brien *et al*., 1987a, Patterson, 2007, O'Brien, 1994, Driscoll *et al*., 2002), where census figures declared the world population of *P. l. persica* to have been reduced to around 18 individuals (Paulson, 1999, Driscoll *et al*., 2002, Gilbert *et al*., 1991, Shankaranarayanan *et al*., 1997, Singh and Gibson, 2011, Wildt *et al*., 1987). The population has gradually been recovering and, positively, the recent census of the Gir forest (2015) reported a promising increase in numbers of Asiatic lions to at least 523 individuals from a recorded 411 in 2010, although no assessment of the genetic viability is given in the study (Singh and Gibson, 2011, Guardian, 2015).

Nevertheless, the geographic isolation of the population generates a risk of extinction if an unpredictable or major natural disaster occurs in the region, which could potentially destroy the entire wild population in one catastrophe (Frankham, 1995b, Banerjee and Jhala, 2012).

1.5 Establishment of the EEP population of *Panthera leo persica*

In the mid 1970's, in an attempt to protect animal numbers, wild lions were captured from within the Gir Forest, and placed into Indian sanctuaries as a monitored population (Dorman, 2009). From this captive population, lions were donated to breeding programmes on both sides of the Atlantic, the Species Survival Plan (SSP) in America and the European

Endangered Species Programme (EEP) in Europe. These initial populations were established in the 1980's, however there were suspicions raised that some of the individuals may have descended from African/Asian hybrids. Genetic testing was carried out, and whilst Gir Forest lions were shown to be monomorphic at all 46 allozyme loci tested, polymorphism was observed at three loci in the SSP population; the allele variants observed within these Asiatic lions had previously only been observed in African lions (O'Brien *et al*., 1987a). The programmes on both sides of the Atlantic were temporarily discontinued (O'Brien *et al*., 1987a, O'Brien *et al*., 1987b). To date, the SSP in America has yet to receive new purebred Asiatic lions from India, which are needed to form a new founder population for breeding in zoos on the American continent (O'Brien *et al*., 1987a, Ashraf *et al.*, 1993).

However, the captive breeding programme was re-established within Europe in the early 1990's when nine lions, believed to be pure-bred, were transferred from India to three European establishments; namely Zurich Zoo, London Zoo and Helsinki Zoo. These nine lions formed the founder population of the EEP. Whilst one of these transferred lions was one of the original wild-caught Gir Forest males from the 1970's, the remaining eight were individuals bred in captivity, and their ancestry can be traced to 11 of the original group of wild caught founders. As such, this population is distinctive in that the 'genetic' founders of the population *i.e.* the sole providers of genetic material to the EEP population (*n*=9) possess a documented pedigree history tracing back to the wild-caught individuals, which are traditionally referred to as founders (*n*=12). Figure 2 illustrates the relationship between the wild-caught founders and the individuals used to establish the EEP population.

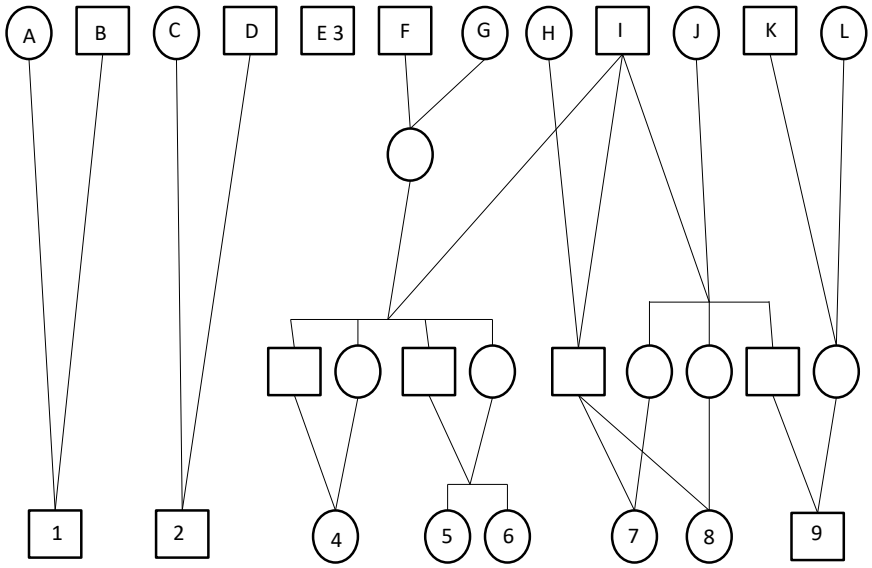

Figure 2. Schematic of pedigree of EEP founders in relation to wild-caught ancestors. EEP founders represented by the numbers 1-9 and the wild- caught ancestors are represented by the letters A-L. Males are symbolised by squares; females by circles. The male labelled 'E 3' is considered both a wild-caught ancestor, and an EEP founder. Individuals labelled 4-9 can all be traced to one common grandfather (I) and individuals 4-8 are the offspring of full-sibling or half-sibling matings. Data adapted from Dorman (2009).

In 2009, the EEP population of Asiatic lions stood at 93 living individuals across 34 sites. The presence of the historic pedigree data allows a more accurate analysis of F-values (which give an indication of the level of inbreeding) and mean kinship values for the EEP population, however with regards to a true representation of genetic variation captured within the establishment of the EEP population, genetic analysis must only concern the nine transferred individuals, in effect treating these as the founder population.

1.6 Captive population in India

There exists a captive population of *P. l. persica* in sanctuaries and zoos in India, with the main wild population found in Gir National Park in Western India (Figure 1). Whilst this population is managed carefully, being the sole population of *P. l. persica* remaining, they are also allowed to roam as wild as possible, and, as such, are often used as the benchmark

for demographic evaluation of the species (Banerjee and Jhala, 2012). The zoo population (e.g. Hyderabad, Sakkarbaug) tends to be used for any biological analysis due to the ease of obtaining the samples from captive individuals.

In the development of the microsatellite markers used in this research, the authors have provided characterisation data for each marker on a population of *P. l. persica* from within captive Indian zoo populations (Gaur *et al*., 2006, Singh *et al*., 2002). Polymorphism was observed at the 27 tested loci (mean observed heterozygosity=0.74, standard deviation=0.27, range=0.05-1) with between 2 and 11 alleles recorded at the loci tested (mean=5, standard deviation=2.15). This suggests that the historic bottleneck documented previously did not reduce variability to an unrecoverable level, as both the EEP and Indian captive populations were formed after this event. This characterisation data does however allow a direct comparison of the variability observed in the captive Indian populations and the variability captured in the nine founders donated to establish the EEP population.

1.7 Aims of the research

In this research, microsatellite markers were used to establish the levels of genetic variation in the nine founders of the EEP population and a selection of contemporary samples. This microsatellite data was compared with published variability data from the Indian captive population (Gaur *et al*., 2006, Singh *et al*., 2002). Sequences of mtDNA (cytochrome *b* and D-Loop) for both the founders and contemporary samples were established and aligned with published sequences. Finally, a review of the pedigree of these nine founders using studbook data and PMx population management software was carried out.

2. Materials and Methods

2.1 Samples

Samples (bone, museum skin samples and tissue) from the nine deceased EEP founder individuals were sourced from zoos and museums within the EEP (Table 2). A further seven tissue samples suspended in ethanol from within the living EEP population were obtained to ascertain any variation which may have been propagated through the generations. The founder samples were sourced from the individuals represented by the numerical designations 1-9 in Figure 2. The contemporary samples represent 7.5 % of the current EEP living population; the sample availability was restricted by access to living lions and relied on the cooperation of zoos providing samples.

Table 2. Summary of sample types used in this study for founder samples. *bone samples were discontinued in the process at a later stage due to continued failed repeated PCRs and experiments continued with the skin samples.

Sample ID	Sample type	Storage before receipt	Storage after receipt
1	Bone	Museum	Refrigerator
2	Bone	Museum	Refrigerator
3	Tissue in ethanol	Refrigerator	Freezer
4	Tissue in ethanol	Refrigerator	Freezer
5	Tissue in ethanol	Refrigerator	Freezer
6	Tissue in ethanol	Refrigerator	Freezer
7	Skin and bone*	Museum	Refrigerator
8	Skin and bone*	Museum	Refrigerator
9	Tissue in ethanol	Refrigerator	Freezer

2.2 Extraction

Tissue extraction was carried out using QIAGEN DNeasy® Blood and Tissue Kit following the manufacturer's protocol (QIAGEN). DNA from bone and skin samples was extracted using an in-house decalcification and digestion method prior to QIAGEN DNeasy® protocol as follows: 50 mg bone powder or museum skin (chopped with a clean scalpel into pieces <1

mm^2) were incubated overnight at room temp in 1 mL 0.5 M EDTA (Ethylenediaminetetraacetic acid) followed by a 1 mL double-distilled water (ddH_20) wash. 360 µl ATL (Qiagen DNeasy® kit pre-prepared buffer), 40 µl Proteinase K (>600 mAU/ ml) and 10 µl 1 M DTT (Dithiothreitol) were added to the sample which was incubated under agitation at 55 °C for a further 24 h. 400 µl AL (Qiagen DNeasy® kit pre-prepared buffer) was added and incubated at 70 °C for 30 min. 400 µl 100% Ethanol was added before completing the extraction with the QIAGEN DNeasy® protocol. Multiple extractions were carried out on bone and museum skin samples to allow for repeated genotyping at all loci.

2.3 Visualisation of genomic DNA

After extraction samples were visualised on 2% agarose (Fischer Scientific: Genetic Analysis Grade) stained with GelRed™ (Biotium; 10,000x in DMSO). Samples were loaded to the gel mixed with 6x TriTrack™ DNA loading dye (Fermentas). Products were separated using Bio-Rad electrophoresis gel tanks at 100 volts. One lane on each gel was used for PCR Ranger 100 base pair ladder (Norgen) to allow approximate quantification; however, the visualisation of a high molecular weight band of any intensity was interpreted as a successful extraction.

2.4 PCR amplification

2.4.1 Microsatellite Analysis

Individuals were genotyped using 12 species-specific published microsatellite markers (Gaur *et al*., 2006, Singh *et al*., 2002). These markers were namely Ple23, Ple24, Ple51, Ple55, Ple57, Ple21, Ple34, Ple53, Ple58, Ple62, Ple65, and Ple251. The primer sequences for these markers are provided in Appendix 1. Products were amplified in 25 µl reactions using illustra™ puReTaq Ready-To-Go PCR beads (GE Healthcare), 1-30 ng template DNA, and 0.4 µM of each primer in single-plex reactions. The amplification protocol solely differed in the number of cycles, which was dependent on the source DNA; tissue samples were subjected

to 30 cycles; bone and museum skins required 35 cycles of PCR. The protocol was as follows: denaturation at 94 °C for 3 min, 30 or 35 cycles of 94 °C for 30 sec, 58 °C for 20 sec, 72 °C for 1 min, with a final extension of 72 °C for 30 min. PCR amplification was carried out using a Techne TC-5000 thermal cycler. Repeated PCR amplifications were attempted on bone and skin samples (a minimum of six times per sample). The failure of an individual to amplify in any of the repeated tests excluded this individual from the data for that specific locus; genotyping data obtained from at least one successful test was included in the analysis.

2.4.2 Sequencing

Sequencing reactions were carried out using published primer sequences and protocols for both cytochrome *b* (Bertola *et al.*, 2011) and mitochondrial D-Loop (Shankaranarayanan and Singh, 1998). The primer sequences are provided in Appendix 2. The cytochrome *b* sequence had a target region of 489 base pairs, and the D-Loop targeted 990 base pairs. Products were amplified in 25 µl reactions using illustra™ puReTaq Ready-To-Go PCR beads, 10-30 ng template DNA, and 0.4 µM of each primer. For cytochrome *b* sequencing, samples were initially denatured at 94 °C for 4 min; 35 cycles of denaturation at 93 °C for 20 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec; final extension at 72 °C for 10 min. For D-Loop sequences, samples were initially denatured at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 15 sec, annealing at 48 °C for 15 sec and extension at 72 °C for 1 min; a final extension step of 72 °C for 7 min.

2.5 Post-PCR visualisation and quantification

Samples were separated using 2% agarose gel and each gel was stained with GelRed (Biotium; 10,000x in DMSO). Products were separated using Bio-Rad electrophoresis gel tanks. One lane on each gel contained PCR Ranger 100 base pair ladder (Norgen) to confirm amplicons of expected size had been amplified. Gels were then visualised using Bio-Rad Gel

Doc™ EZ Imager and Bio-Rad Image Lab 4.1 software which allowed quantification of the amplified product through the use of the PCR Ranger ladder.

2.6 Purification and dilution

Sequencing samples which were observed to have amplified a specific band of expected size (using gel electrophoresis) for cytochrome *b* (n=12, founders=5, contemporary=7) were purified in-house using illustra™ ExoStar™ purification kit (GE Healthcare). One sample was re-quantified (using Bio-Rad Gel Doc™ EZ Imager and Bio-Rad Image Lab 4.1 software) using agarose gel electrophoresis band intensity in relation to the ladder intensity (of known concentrations) to ascertain the approximate dilution factor that was occurring during the purification process. This dilution factor was then applied to all initial concentrations of amplified products to establish the concentrations of all samples after purification. These concentration values were used to calculate the amount of amplified product to be added to PCR grade water to make a sample with a concentration of 5 ng/µl.

D-Loop sequences (n=16, founder=9, contemporary=7) and founder cytochrome *b* sequences which failed to produce a visible band on an agarose gel (n=4) were purified and diluted as part of the 'a-la-carte' service offered by Eurofins in an attempt to improve the quality of the sequence obtained.

2.7 Capillary Electrophoresis

2.7.1 Microsatellite Analysis

Forward primers were 5' fluorescently tagged for automated genotyping (see Appendix 1). Alleles were separated using capillary electrophoresis (CE) in the ABI3730 Genetic Analyser (Applied Biosystems®) carried out by NERC Biomolecular Analysis Facility (Sheffield, UK).

2.7.2 Sequencing

Samples for sequencing underwent CE at Eurofins using either their 'a-la-carte' service for raw PCR products, or, for products purified in-house, their 'Ready to load' service.

2.8 Post CE Analysis

2.8.1 Microsatellite Analysis

Raw data was received electronically from the external supplier. Applied Biosystems® Peak Scanner™ Software (v.1.0) was used to establish peak data. The size of each amplicon in base pairs (to 2 decimal places) was recorded and FlexiBin (Amos *et al*., 2007) was used to bin alleles, a program which uses a Microsoft Excel Macro to explore the possible and/or likely binning patterns. The software accounts for the variances between mobility and the true fragment size, assuming each di-nucleotide repeat contributes between 1.7 and 2.3 base pairs to the strand (+/- 0.3 base pairs from the expected '2') which is given as the 'effective repeat unit length'. However, to account for the slight variation in mobility between strands and runs, another figure, termed 'offset' is explored. The combinations of values for offset and effective repeat unit length are explored in order to evaluate the goodness of fit for each allele into a bin.

2.8.2 Sequencing

Sequencing data was analysed using BioEdit (Hall, 2013). The sequences generated were aligned with each other using ClustalW (Thompson *et al*., 1994) using full multiple alignment, bootstrap NJ tree with 1000 bootstrap values, and against other published sequences sourced from Bertola *et al*. (2011). Mega 5.0 (Tamura *et al*., 2011) was used to create a maximum likelihood tree using the best-fit model recommended by the MEGA software (Hasegawa-Kishino-Yano model). All other options were set to default parameters.

2.9 Analysis of studbook

Studbook data was inserted into PMx population management software (Lacy *et al*., 2011). As some pedigree knowledge of the nine EEP founders is known, all individuals in the pedigree (up to and including the historic wild-caught founders) were essential to the effective and correct kinship analysis of the population using PMx software. However, in terms of captured genetic variation, mean kinship and mean inbreeding, the EEP founders were used as the founding generation, as the sole providers of the genetic material. The program was used to calculate the following data:

(i) Representation Values: proportion of genes in the descendant population that derive from that particular founder (maximum value of 0.5)

(ii) Contribution Values: number of copies of a founder's genome that are present in the living descendant population, where each offspring contributes 0.5, each grand-offspring contributes 0.25. The higher the number, the more copies of founder genomes are present in the descendant population.

(iii) Allele Retention Values: probability that a random gene in a founder individual exists in at least one individual in the living descendant population: (maximum value of 1).

The Mate Suitability Index (MSI) within the PMx software was used to generate a composite score suggesting the most genetically beneficial matings integrating four genetic components:

(i) change in genetic diversity of the population by the production of the potential offspring,

(ii) difference between the parental male and female MK values,

(iii) F-value of the potential offspring, and

(iv) any unknown ancestry.

This composite score ranges from 1-6; the lower the number, the more suitable the mating. An index score of 4 or more infers an increasing detriment to the population as a result of the mating. Outside of this numerical range there is also the dash symbol '-' which states that the mating would be 'very highly detrimental'.

2.10 Statistical calculations

Hardy-Weinberg equilibrium (HWE) and Linkage Disequilibrium (LD) was calculated using Genepop v.4.2 (Raymond and Rousset, 1995). Observed (H_O) and expected (H_E) heterozygosity were calculated manually. The number of individuals observed to be heterozygous at each locus was divided by the total number of individuals genotyped at the locus to produce the observed heterozygosity value (H_O). The observed numbers of alleles at each locus are converted to a proportion and the expected heterozygosity (H_E) can be calculated using the formula below:

$$H_E = \left(1 - \sum_{i=1}^{m} p_i^{\,2}\right)$$

where p_i is the frequency of allele 'i' and m is the number of alleles at the locus

In order to test the suitability of a regression analysis, the distribution of F-values calculated for the historic population were tested using SPSS (version 23.0, IBM Corporation, 2015) to determine whether there was a significant difference from a normal distribution. Linear regression between population F-values and generation time was carried out using SPSS (version 23.0, IBM Corporation, 2015), with residual plots being checked for random distribution of data and to check the goodness of fit of the regression.

3. Results

3.1 Microsatellite Analysis

Genomic DNA extraction was visualised successfully for all samples extracted. However, due to repeated PCR failures for the bone samples for individuals 7 and 8, only the skin extractions for these individuals were continued (Table 2). A summary of numbers of repeated PCR and resulting successful amplifications for each individual is provided in Appendix 3. The characterisation data obtained here for the EEP founders and EEP contemporary samples are compared to the figures provided for the captive Indian population from Gaur *et al*. (2006) and Singh *et al*. (2002) (Table 3). Full genotypes for the founders are provided as microsatellite fragment sizes in Appendix 4.

Table 3. Genotyping summary for the EEP founders in comparison to the captive Indian population. N= number of individuals successfully genotyped, K= number of observed alleles, H_O= Observed heterozygosity, H_E= Expected heterozygosity. *some repeated amplifications failed **one individual amplified two alleles in some repeats (6/16 tests) and showed allelic drop-in or drop-out of one or the other allele in the other repeats. Indian data sourced from Gaur *et al*. (2006) and Singh *et al*. (2002).

		Marker (Ple)											
		23*	**24***	**51**	**55***	**57***	**21***	**34***	**53**	**58***	**62**	**65**	**251**
EEP Founders	**N**	9	9	9	9	9	8	7	9	8	9	9	9
	K	1	2**	1	2	2	1	1	1	1	1	1	1
	H_O	-	0.11	-	0.78	0.33	-	-	-	-	-	-	-
	H_E	-	0.11	-	0.48	0.50	-	-	-	-	-	-	-
Contemporary population	**N**	7	6	7	7	7	7	7	7	7	7	7	7
	K	1	1	1	2	2	1	1	1	1	1	1	1
	H_O	-	-	-	0.43	0.71	-	-	-	-	-	-	-
	H_E	-	-	-	0.46	0.46	-	-	-	-	-	-	-
Indian population	**N**	15	15	15	15	15	22	22	22	22	22	22	22
	K	8	6	8	11	7	6	5	6	4	6	5	4
	H_O	1.00	0.06	0.86	0.86	1.00	0.95	0.57	0.94	0.63	0.68	0.84	0.84
	H_E	0.58	0.41	0.85	0.71	0.58	0.68	0.61	0.77	0.62	0.77	0.75	0.60

In the founder samples, five of the markers (Ple51, Ple53, Ple62, Ple65 and Ple251) produced a consensus homozygous genotype in all individuals tested. Another four of the markers (Ple21, Ple23, Ple34 and Ple58) also produced a consensus homozygous genotype

in all successful amplifications, however not all repeated amplifications were successful in generating a profile. Within these four markers there were also some failed amplifications in some individuals, despite repeated tests; namely Marker Ple34 in individuals 6 and 8, and individual 2 at markers Ple21 and Ple58.

Marker Ple24 produced a homozygous genotype in all individuals, except individual 8. Bi-allelic heterozygosity was observed for this individual sample in some of the repeats (6 of 16 amplifications) and the remaining ten amplifications demonstrated drop-in or drop-out of one or other of the alleles. The H_O for this marker is 0.11 (H_E= 0.11).

Markers Ple55 and Ple57 showed bi-allelic heterozygosity with H_O figures of 0.78 and 0.33 respectively (H_E= 0.48 and 0.5, respectively). However, there were some failed repeated amplifications, and drop-in or drop-out was observed in two of the six successful repeats at marker Ple55 in individual 8.

No novel alleles were observed in the contemporary samples. All markers were successfully amplified for all individuals, aside from marker Ple24 in one individual, despite repeated attempts. The samples for the contemporary members of the EEP population were all sourced from post-mortem tissue which had been freeze-stored suspended in ethanol, so this success rate was as expected given the more ideal sample storage conditions for DNA extraction (Wang *et al*., 2013, Wheeler *et al*., 2017). Amplifications were repeated across markers and individuals to confirm the results. Markers Ple55 and Ple57 were the only markers which produced bi-allelic profiles in this sample set with H_O figures of 0.43 and 0.71 respectively (H_E= 0.46 and 0.46 respectively).

Hardy-Weinberg Equilibrium was tested and accepted for markers Ple55 and Ple57 in both founders (p= 0.17 and 0.50 respectively) and contemporary samples (p= 1.00 and 0.44 respectively). HWE was unable to be calculated for Ple24, as only one individual was potentially bi-allelic at this locus (Individual 8). Given the possibility for allelic drop in/drop

out at locus Ple24, LD was only calculated between loci Ple55 and Ple57 and was tested and accepted (p=1 and 0.57 respectively for founders and contemporary).

In the EEP founder population, nine of the 12 markers utilised amplified only one allele across all individuals tested. In the remaining three markers, the levels of observed heterozygosity were lower in the EEP population than in the Indian captive population, and were only observed in bi-allelic form (Table 3, Figure 3). In the contemporary samples only two of the markers were bi-allelic, both with observed heterozygosity figures lower than in the Indian population.

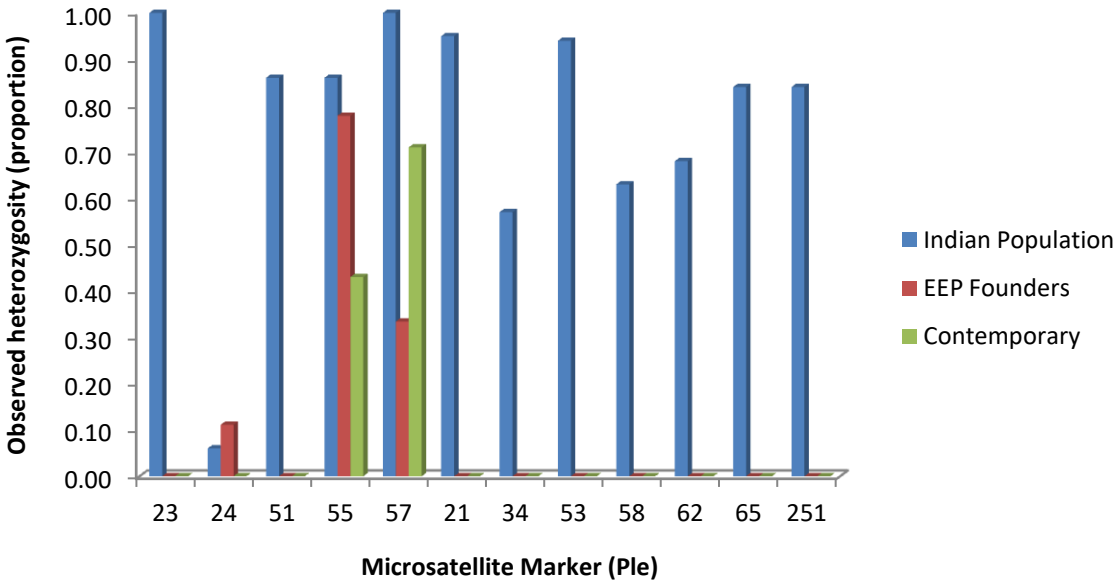

Figure 3. Bar chart comparison of observed heterozygosity in EEP founders, EEP contemporary samples and Indian captive populations. Data for Indian population obtained from Gaur *et al*. (2006) and Singh *et al*. (2002).

3.2 Sequencing

3.2.1 Cytochrome *b*

Cytochrome *b* sequences were successfully generated for eight of the nine founders and all seven of the contemporary individuals for the cytochrome *b* fragment. Six of the founders and five of the contemporary sequences were confirmed by both sequences (forward and reverse); the remaining four samples were only successful in amplifying the reverse strand. The sequences successfully obtained ranged from 401 bases to 468 bases in length. Aligning these samples against all 53 *Panthera* sequences provided by Bertola *et al*. (2011), a Maximum Likelihood Tree was produced which aligned these samples with the four individuals sourced from the Gir Forest population by the aforementioned study (Figure 4). An example cytochrome *b* sequence obtained in this study is provided in Appendix 5.

3.2.2 D-Loop

Forward and reverse sequences were successfully generated for the D-Loop for four of the founders and five of the contemporary samples. The sequences successfully obtained ranged from 806 bases to 860 bases in length. The sequences were compared to two mitochondrial sequences of *P. l. persica* available on Genbank (Accession numbers KU234271.1 and KC834784). A summary of the differences are shown in Table 4. An example D-Loop sequence obtained in this study is provided in Appendix 5.

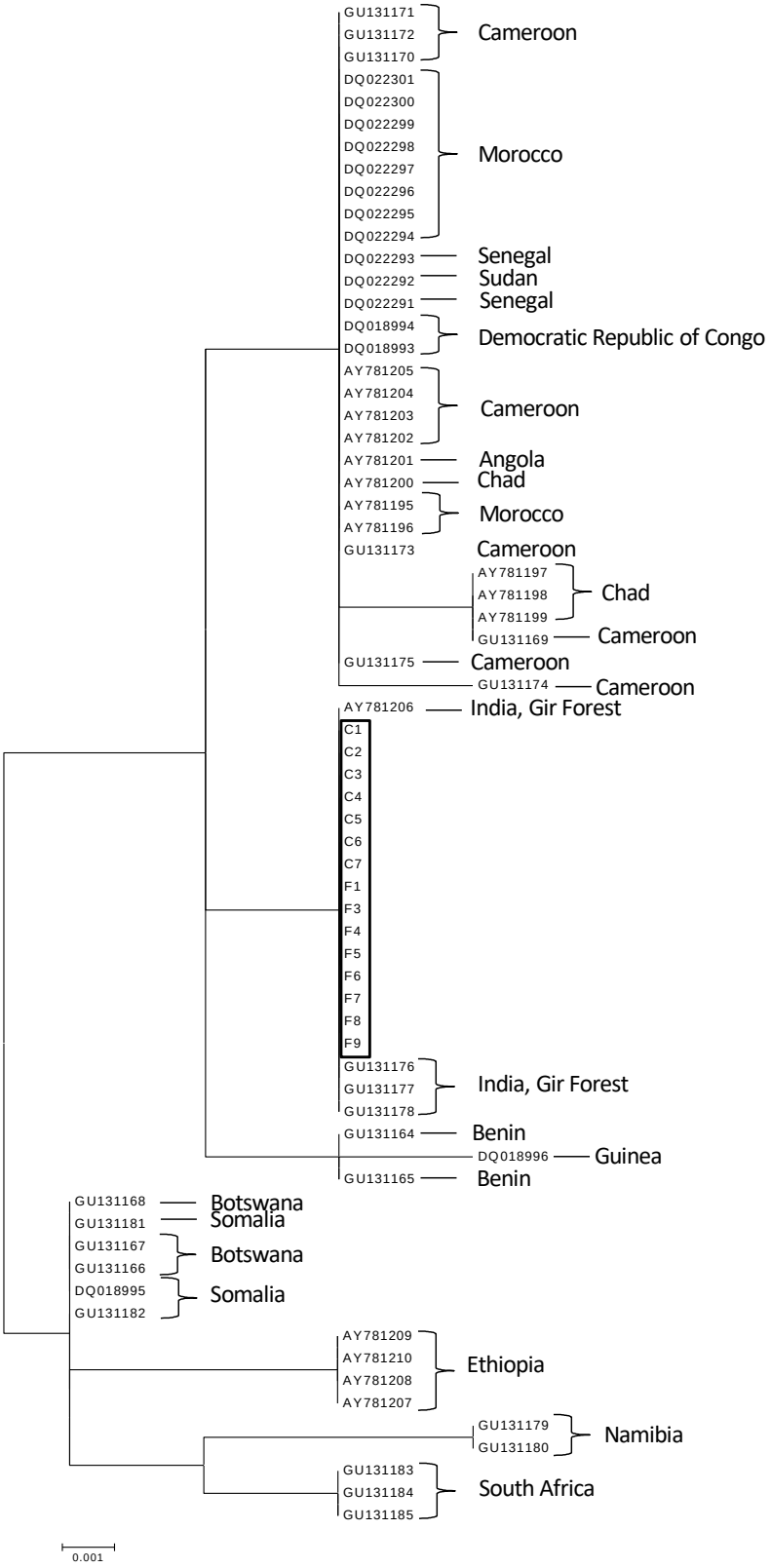

Figure 4. Maximum Likelihood Tree showing alignment of cytochrome *b* sequences. Sequences from this study (boxed in black) were aligned against 53 *Panthera* sequences sourced from Bertola *et al*. (2011).

Table 4. Summary of nucleotide changes in D-Loop sequences (EEP against published Indian sequences). Alignment contains two published *Panthera leo persica* sequences from Indian lions (Accession numbers: KU234271.1 and KC834784) and four founder (F) sequences and 5 contemporary (C) samples from this study. Nucleotide number denotes the nucleotide location on the reference sequence (KU234271.1). '.' denotes base identical to reference sequence. '-' denotes missing data. Bases are symbolised by the accepted nomenclature of A=Adenine, T=Thymine, C=Cytosine and G=Guanine.

Position	31	31	32	32	34	34	34	34	34	34	34	35	35	35	35	35	35	35	35	37	38	46	43	54	54	55	55	55	64	65
	18	19	20	29	33	44	45	46	67	78	89	90	01	23	34	55	56	57	71	15	54	43	31	94	55	57	84	44	58
P.l. persica (KU234271.1)	G	T	A	C	C	A	C	A	C	G	T	A	C	A	C	G	T	A	C	C	C	A	T	T	T	T	T	A	T	C
P.l. persica (KC834784)	A	C	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	G	C	C	C	C	-	-	C	T
F4	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
F5	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
F6	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
F9	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
C1	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
C2	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
C3	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
C4	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T
C5	A	C	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	G	C	C	C	.	-	-	C	T

3.3 Studbook data analysis

Using the 'European Studbook for the Asiatic Lion Number 5' (2007-2009) the pedigree for the nine EEP founders was established from the date where the 12 historic founders were caught from the wild within the Gir Forest and placed into Sakkarbaug Zoo, Junagadh. From the date of capture for the wild-caught individuals (1972-1990) to the transfer of the EEP founders to establishments within Europe (namely London, Helsinki and Zurich) in 1990-1992 there were 10 matings traced back to nine of the wild caught founders. These matings produced six of the nine EEP founders; the final EEP founders were sourced directly from the original wild caught population, two having been born in captivity with unknown sires (Figure 2). It can be seen that there are numerous issues with the pedigree of the nine EEP founders. Firstly, six of the individuals (4-9 on the schematic) share a second generation common ancestor, namely their grandfather- individual I. Importantly, these individuals include the entire female EEP founding population, along with one of the four males. Secondly, three of the individuals are the offspring of full-sibling matings (4-6), and a further two are the offspring of half-sibling matings (7 and 8). All five of these individuals are the founding female population of the EEP, and as such the female ancestors to the EEP

population all possess a high F-value, signifying that they are inbred animals. Individuals 4-6, as the offspring of full-siblings, have an F-value of 0.25. Individuals 7 and 8, as the offspring of half-sibling matings, have an F-value of 0.125. Assuming non-relatedness of the initial 12 wild-caught founders, three of the four males transferred to the EEP share no common ancestors with these inbred females, allowing two of the establishments to receive mating groups which had no relatedness. The final male (9 on the schematic) shared two common ancestors (I and J) with females 7 and 8 (kinship value of 0.0938), and as such was transferred with one of the females (4) with whom he shared just one common ancestor (I) (kinship value of 0.0625).

It also has to be considered that individuals B and D (the unknown sires of individuals 1 and 2) may already be represented by one or more of the other males in this schematic (i.e. Individuals E, F, I or K), which would further compound any issues regarding the relatedness of these EEP founders.

The stated N_e for this population is 41.14. The figures calculated in this research for gene diversity (GD; 0.8658), mean kinship (MK; 0.1342) and mean inbreeding level (F; 0.0489) for the living population match the figures published in the 'European Studbook for the Asiatic Lion – Number 5'. The individual MK for each living individual against the rest of the population ranges between 0.0967 and 0.1538 (mean= 0.1342, standard deviation=0.011, data not normally distributed, Shapiro-Wilk W = 0.957, $p<0.05$). However, individual MK values represents mean kinship of that lion to all other living lions in the population. As lions mate in a pairwise fashion this number does not give a true representation of the kinship of any given breeding pair. If we consider pairwise kinship values across the 93 living individuals, 33.7% of the comparisons are above the population MK value (>0.1342) and the remaining 66.3% of comparisons are below the population MK value (<0.1342) (range 0.0313-0.3428). Of the comparisons above the MK value, 25.9% of the values are in

the range of 0.1343-0.1999, 6.2% are in the range 0.2-0.2999 and 1.6% are in the range above 0.3 (Table 5).

Table 5. Pairwise kinship comparison (n=4278) of living individuals in relation to population MK value (0.1342).

	Pairwise kinship value	Number	Percentage
≤ population MK value	0.0000-0.1342	2836	66.3
> population MK value	0.1343-0.1999	1108	25.9
	0.2000-0.2999	265	6.2
	0.3000-0.4999	69	1.6
	Total	4278	100

PMx provides F-values for the current living population (n=93) in the range of 0 to 0.1270 with a mean of 0.0489 (standard deviation=0.0403). The F-value range for the entire historic population (n=336) is 0 to 0.306 with a mean value of 0.0536 (standard deviation=0.0466). The data are not normally distributed for either the current living population (n=93, Shapiro-Wilk W=0.89, $p<0.001$) or the entire historic population (n=336, Shapiro-Wilk W=0.86, $p<0.001$). The highest F-value recorded in an individual was 0.3086; this cub died on the same day as birth.

A linear regression found a positive correlation between generation number (from ancestral founders) and individual F values for the entire historic population (n=336) (r^2=0.349, F=178.65, $p<0.001$, Figure 5). Residual plots for this analysis showed a random distribution of data points, justifying the fit of the regression to this dataset. From Figure 5 it can be seen that the group of lions classed as generation 5 have an F-value of just below 0.10, higher than the provided population-wide figure of 0.0489, due to the presence of lower F numbers contained within the population wide figure from the surviving individuals from earlier generations.

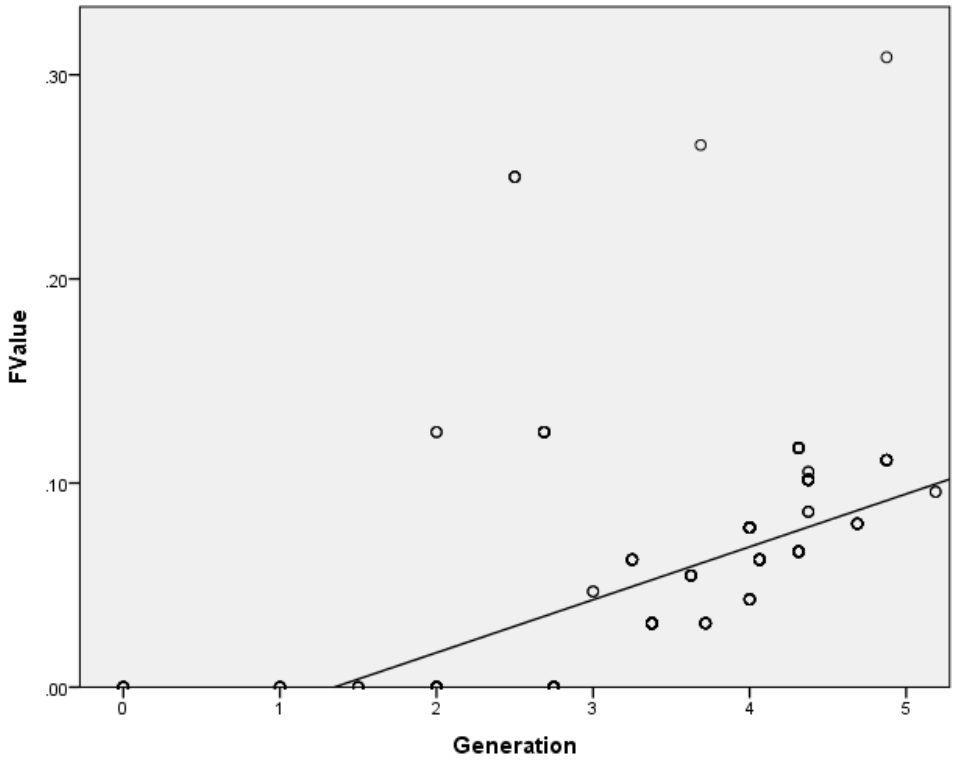

Figure 5. Linear regression of individual F-values (FValue) against generation number (Generation) for entire historic population (n=336).

With regards to GD, two separate analyses were carried out using PMx, one using the full studbook data, back to the wild-caught founders; the second using the studbook from the inception of the EEP population, in other words, without the EEP ancestry included. The entire studbook produces a GD figure of 0.8658, which means that 86.58% of the GD of the original founders has been captured by the current population. Using the EEP as the population founders, the figure increases to 0.9083, meaning that 90.83% of the GD of the nine EEP founders has been captured by the current living population. However, there have only been 3.75 generations since the EEP was founded, so this represents a loss of GD of nearly 10% over less than 4 generations (2.4% per generation). Since the wild-caught founders there have been 5.34 generations, representing a loss of nearly 14% over this period (2.5% per generation).

The representation of particular founders, in both the EEP founder population and the EEP ancestors can be shown using data on Representation Values, Contribution Values and

Allele Retention Values from PMx. The values for these data are provided for the 12 wild-caught population ancestors and the nine EEP founders (Table 6). As Individual I is a common ancestor to all five EEP founding females, he is therefore the common ancestor to all 93 individuals in the EEP population and as such, his living descendants encompass all living individuals. This individual also has the highest Contribution Value which relates to the number of descendants.

Table 6. PMx data demonstrating representation, contribution, allele retention and living descendants for all 12 wild-caught founders and the 9 EEP founders. (see text for explanation of terms) *Individual E/3 is the same individual although is included within both populations**.**

	Founder	Representation	Contribution	Allele Retention	Living Descendants (/93)
Wild –Caught Individuals	A	0.0726	6.75	0.50	57
	B	0.0726	6.75	0.50	57
	C	0.0454	4.22	0.46	46
	D	0.0454	4.22	0.46	46
	E*	0.2292	21.31	1	76
	F	0.0738	6.86	0.37	85
	G	0.0738	6.86	0.37	85
	H	0.0512	4.77	0.36	76
	I	0.2587	24.06	0.93	93
	J	0.0600	5.58	0.52	80
	K	0.0087	0.81	0.20	17
	L	0.0087	0.81	0.18	17
EEP founders	1	0.1452	13.50	1	57
	2	0.0907	8.44	0.92	46
	3*	0.2292	21.31	1	76
	4	0.0349	3.25	0.77	17
	5	0.1828	17	1	66
	6	0.0773	7.19	0.98	32
	7	0.1499	13.94	0.99	66
	8	0.0551	5.13	0.88	28
	9	0.0349	3.25	0.77	17

Of the EEP founders, the number of living descendants range from 17-76 individuals and the allele retention figures in six of the nine founders is greater than 0.9. Again, it has to be remembered that individuals B and D (the unknown sires of individuals 1 and 2) may be represented by one of the other males already in this pedigree and would further complicate the figures presented here.

Potential matings within the population were assessed with the MSI within the PMx software. Currently, in the EEP (with the removal of one sterile male) there are 35 males and 57 females, so there are 1995 potential matings. Of these 1995 pairings, 965 (48.37%) are classed as 'very highly detrimental matings' ('-'); 913 matings (45.76%) are classed as 'very detrimental' (280; 14.04%), 'detrimental' (81; 4.06%) or 'slightly detrimental' (552; 27.67%). 117 matings (5.86%) are in the beneficial end of the MSI scale, with 67 (3.36%) and 50 (2.51%) being classed as either 'slightly' or 'moderately' beneficial respectively.

In assessment of the cub survival rates within this captive population it was discovered that since the establishment of the EEP population in 1990 over 50% of the offspring have died within 30 days of birth (Dorman, 2009). During 2007-2009, there were a total 57 cubs born into the EEP population; 18 of these cubs born died within the first 24 hours, a further 12 survived less than five days, and another four individuals did not survive the first 20 days. The total cub mortality rate was 68% during the 2007-2009 period.

3.5 EEP management

In reviewing the studbook, matings which have occurred since the inception of the EEP can be examined. There are specific examples which have been analysed in detail. In the first example, a male and female (kinship 0.1016) mated at Besancon, France giving birth to two daughters (F-value 0.1016). As the average age of females at first reproduction being just over 5.5 years, offspring under this age are not considered to be 'at risk' of being impregnated by male relatives. However, in Besancon one of the daughters was

impregnated by her father during her adolescence, becoming the youngest female to give birth at 2 years, 3 months and 7 days. Both daughters were transferred away from their father as soon as the pregnancy was discovered. The offspring did not survive 24 hours, being the individual with the highest F-value in the population at 0.3036. Ultimately, no decision was required on whether the offspring should be medically euthanized as its death was almost immediate after birth, possibly due to the level of inbreeding, although this cannot be stated categorically.

In the second case, two of the EEP founders (received by Helsinki) produced four litters (cubs=13). One of these cubs, a female, was transferred via London to Paignton, where she mated with a resident lion (kinship- 0.0313). These matings produced a total of six litters (cubs=14). One of these female cubs was transferred in June 2000 to Boissiere, where she was joined by her grandfather in May 2001. The pair mated producing an initial litter of two viable offspring (F= 0.1250). The matings continued and three single cub litters were born and did not survive the first few weeks. It was only whilst pregnant for the fourth time with two more of her grandfather's cubs was the female transferred to another establishment. Out of the seven cubs born from these five litters only three have survived to the current population and viable age.

4. Discussion

4.1 Microsatellite Analysis

This research has shown that the founding EEP population demonstrates excessive homozygosity at the markers tested, confirmed by the contemporary samples. It could be suggested that the well documented reduced variability in Asiatic Lions as a result of the historic bottlenecks or founder effect has a role in these findings (Barnett *et al*., 2006b, Burger *et al*., 2004, Antunes *et al*., 2008, O'Brien *et al*., 1987b, O'Brien, 1994, Driscoll *et al*., 2002). Hunting activity by humans at the turn of the 20th Century would also contribute to the reduced variability being present in the surviving individuals (Paulson, 1999, O'Brien, 1994). The genetic paucity of the Asiatic Lion has previously been compared to the lack of variation already observed in the cheetah (*Acinonyx jubatas)* (O'Brien *et al*., 1985, O'Brien *et al*., 1986, Driscoll *et al*., 2002, O'Brien, 1994). Multiple research projects have found excessive homozygosity in the Asiatic lion, using allozymes, minisatellites and microsatellites (O'Brien *et al*., 1987b, Wildt *et al*., 1987, Gilbert *et al*., 1991, Shankaranarayanan *et al*., 1997, Driscoll *et al*., 2002).

However, if the lack of observed variation could be explained by these historic bottlenecks and population crashes, then the Asiatic Lion would show uniform genetic paucity across all pure populations tested. Conversely, recent research using *P. l. persica* samples from the captive Indian population has shown an encouraging level of variation across the microsatellite marker utilised here (Gaur *et al*., 2006, Singh *et al*., 2002). As such, this research allows a comparison between the variation currently present in the captive Indian population and the EEP population. The results clearly show that, in the establishment of the EEP, the variation observed in the Indian captive population was not captured (Table 3, Figure 3). Using identical markers, heterozygosity was observed in all 12 of the markers

used in the Indian population, in comparison to the EEP founders which only showed variability at three of the markers tested, and with only two alleles being observed at these loci. An argument could be made regarding the small sample size used in this research having an effect, but, as the samples tested concern the entire founding population, the sample size is a finite value and cannot be increased. The inclusion of various contemporary samples in the study produced no novel alleles or unexplained variation. It is also unlikely that the excessive homozygosity observed here is due to the phenomenon of null alleles as the homozygosity is observed across multiple markers (Dakin and Avise, 2004).

The papers from which these markers were sourced differed in their descriptive statistics, with one of the papers calculating HWE for the markers, and the other making no mention of this (Gaur *et al*., 2006, Singh *et al*., 2002). The paper which covered this calculation found deviation from HWE in marker Ple62 ($p<0.001$) but all the other markers are not shown to deviate from HWE. Whilst the calculations completed here do not show a deviation from HWE, this finding must be taken with caution, as only two loci were found to be variable and only in a bi-allelic state.

4.2 Sequencing

Sequencing of two regions of mtDNA confirmed the lack of variation observed in the microsatellite analysis. Aligning the samples which were successfully sequenced for the cytochrome *b* region, they can be seen to be grouped with other previously sequenced Gir Forest lions (Figure 4). As the full Gir Forest population has not been sequenced at this region, then it is impossible to ascertain whether the homozygosity in this region is endemic to the sub-species, and therefore may be attributable to the bottlenecks documented in this population. In other words, the reduction of numbers to below 20 may have reduced maternal variation to one mitochondrial haplotype within the sub-species, and therefore may not be unique to this captive population. However, it should be noted

that cytochrome *b* is generally used as a molecular tool for species identification, and, as such, variation between individual Asiatic lions would not be expected.

Some variation was observed between published *P. l. persica* D-Loop sequences and the sequences obtained here, specifically 13 nucleotide differences between sequence KU234271.1 and 2 to KC834784 (Table 4). The main difference has occurred through a 14 base deletion in the samples tested in this study from base 343-356 against the reference strands (KU234271.1 and KC834784). A further 2 base pair deletion was also observed at bases 557 and 558 against the reference strands.

4.3 Studbook Analysis

Studbook analysis shows that this reduced variability in the EEP population may be due to matings occurring after the capture of the 12 wild caught individuals, but prior to the donation of the nine EEP founders. The studbook documents matings during this period occurring between full- and half-siblings, in turn producing offspring which were then transferred to establish the EEP population. This resulted in all the founder females and one of the founder males sharing the same grandfather. Despite the limited awareness at the time of the use of genetic information as a conservation tool, in these circumstances, as historic pedigree was available, it appears a disappointment that this circumstance has happened, and the population was founded was using these related and inbred individuals.

It is worth remembering that all this analysis is based on the assumption that the 12 wild-caught founders were unrelated. However, through the unavoidable situation of sourcing the individuals from a wild population in which we are aware of issues stemming from historic bottleneck events and low population numbers, some ambiguity inherently remains regarding relatedness of founders (Russello and Amato, 2004). In this case, there is also the potential further complication of the possibility of the representation of the unknown sires of individuals 1 and 2 amongst the wild-caught founders already included in the

calculations. As the mothers of these cubs were captured whilst gestating, the sires are currently assumed to be separate individuals, however, this assumption may well be incorrect. Although recent and rapid advances in genetic analysis and resources have been made to assess relatedness of founders, in most cases the unrelatedness of founders has to be assumed, as it is impossible to guess at the levels of relatedness with no pedigree information. However, when this assumption is violated it results in an under assumption of relatedness and kinship (Russello and Amato, 2004, Goncalves da Silva *et al*., 2010), although under estimation is better than overestimation (Russello and Amato, 2004, Willis, 1993, Willis, 2001). Whilst it is accepted that making the assumption that founders are unrelated has negligible effect on long term viability, this is normally because the assumption is true, and when there are related founders, there is a rapid increase of inbreeding within the first few generations of captivity (Ivy *et al*., 2009).

However, whilst this previous knowledge of kinship would be assumed to be out of the control of the EEP management programme, there is also evidence of unsuitable matings occurring across the captive population since the inception of the EEP population. The two examples presented above show one mating between a father and daughter (producing an individual with an F-value of 0.31); the other multiple matings between a grandfather and granddaughter before their separation. These two examples demonstrate the issues faced in relation to captive population management. In the first case, the mating between father and daughter could not have been anticipated due to the young age of the daughter, and there was an immediate and effective response, with both the pregnant daughter and her potentially at-risk sister being transferred away from the father to prevent further issues. This is an example of an unpredictable event occurring, but with the correct reaction from the managers of the zoo and studbook. However, in the second example, there was the initial error of transferring the grandfather to the same location as his granddaughter, and then the failure to react firstly, to the initial pregnancy and secondly to the subsequent

single-cub non-viable litters. It was only after the granddaughter fell pregnant for the fifth time by her grandfather was action taken to remove her from his location. This example is direct evidence of mating occurring due to location rather than the best matings for the population as a whole. Whilst it must be accepted that hindsight and scientific advances complicate our modern day view of these situations, it must be also be noted that these examples are taken from a time when studbook management was a key feature in the establishment, propagation and viability of captive populations.

Reassuringly, the package PMx now offers the MSI, providing a scale of mating suitability based on the studbook data, and recommends or opposes particular matings depending on each individual's history, number of ancestors found common to the potential mother and father, and how the potential offspring will change the dynamic of the population as a whole. However, the index is not encouraging for this population, with less than 6% of the potential matings being classed as not detrimental to the population. Indeed, these matings are only classed as 'slightly' or 'moderately' beneficial to the population, and not a single mating is classed as 'very' beneficial to the population. Nearly half (48.37%) of the entire potential matings are described as being 'very highly detrimental (should not be paired, due to high level of kinship of pair)'. So whilst there is now software in place to make recommendations for the mating of individuals in captive populations, the data provided here does not offer confidence in the long term viability of the EEP population. It is key to note that not one of the current EEP member matings are classed as 1 on the MSI scale, which is described as being 'very beneficial to the population'. Also of concern is the fact that MSI is generated on the studbook data alone, and the incorporation of the microsatellite data above would produce a much more severe grading of the situation, due to the excessive homozygosity suggesting closer kinship than the software would assume. If, as mentioned previously, individuals B and D were already represented amongst the wild-caught founders, this would also affect this MSI scale of matings.

It may be suggested that the MSI recommendations made by the PMx software must become a priority regardless of the cost of the transfers of these lions (Dubach *et al*., 2005). Considering 'cheaper' matings at the detrimental end of the scale, motivated by financial pressures, may have longer term cost implications than establishing 'costlier' matings at the lower end of the scale.

The incorporation of genetic data into the PMx software would clearly worsen these recommendations as the software is assuming a level of genetic diversity was present in the founders. As it has been established here, this variation was absent, so this assumption is incorrect. However, due to the homogeneity of the samples, any kinship inference is impossible as the marker based relatedness would suggest a closer relationship that the true genealogical ancestry (Russello and Amato, 2004). In previous studies which have had access to studbook pedigree, and then used genetic data to infer relatedness have produced a misclassification rate of between 40 and 80%, where genetic data produced a profile of relatedness which was genealogically incorrect (Gautschi *et al*., 2003, Ivy *et al*., 2009, Russello and Amato, 2004). However, whilst assumptions surrounding the relatedness of founders can vary, ranging from the 'founder assumption' (all individuals are unrelated), to the 'worst-case scenario' (assuming high kinship amongst founders), research has shown that to maximise genetic diversity it is better to underestimate the kinship of founders, i.e. follow the founder assumption (Goncalves da Silva *et al*., 2010, Ivy *et al*., 2009, Russello and Amato, 2004).

It could be argued that due to the relative homozygosity in the population, all the matings are potentially detrimental to the population, whether due to close kinship in the dyads, or as a result of inbreeding within the population reducing observed variation. However, when the knowledge of the true genealogical relationships between all individuals is known, then the recommendations are based on true kinship. In other words, a dyad might have

identical genotypes across 12 loci, but if there is no close kinship between the pair, then that mating is more beneficial to the population, than a mating involving a dyad who share a profile but through close kinship. It must be remembered that the microsatellite loci tested represent only a small portion of the total genome of an individual.

By carrying out further investigation, it has been demonstrated that the presentation of population-wide mean values for studbook data does not always present the key data in the most meaningful manner. For example, whilst the mean MK value across the whole population is 0.1342, over 33% of pairwise comparisons fall above this value. Indeed, 7.8% of the population provide pairwise kinship values of between 0.2 and 0.3248, which is extremely high. The data is not normally distributed, and stating a population mean is not representative of the population, and is affected by the overrepresentation of smaller individual mean MK values closer to the population mean. In addition, each individual's MK value is also affected by the majority of pairwise comparisons with the rest of the population being close to the mean. In a captive finite population, it is specific pairings that need to be considered, as an individual with a low MK may be suggested to mate with another low MK individual, however, their pairwise kinship may be high. Nevertheless, MK values provide a useful statistic as through minimising mean kinship, gene diversity and the representation of founder genome equivalents are maximised (Russello and Amato, 2004, Goncalves da Silva *et al*., 2010, Ivy *et al*., 2009). It is suggested inclusion in the breeding pool should depend on MK values; if the individual's MK value is higher than the population mean then it should be excluded from further breeding (Goncalves da Silva *et al*., 2010, Russello and Amato, 2004).

The stated N_e for this captive population is 41.14, slightly below the number required for limiting inbreeding depression recommended within the 50/500 rule (Soulé, 1980, Franklin, 1980), and well below the larger number recommended for the maintenance of long-term

evolutionary potential. Given the revisions to this ratio up to 100/1000 (Frankham *et al.*, 2014) there are apparent concerns relating to this low effective population size within this captive population.

The population mean inbreeding figure of 0.489 is not indicative of some of the higher individual F-values, as again, the data is not normally distributed. The presence of low F-values from earlier generations counteracts the larger figures in the later generations, and reduces the population mean accordingly. Again, it is each individual F-value which must be assessed, and not the population mean. The observed relationship between generation number and individual F-values (Figure 5) is not an unexpected relationship as, explained previously, any closed population will eventually become inbred, however, this graph shows that if individuals were grouped purely by their 'birth' generation the mean values would increase with generation number. However, as inbreeding depression has a linear relationship with the population inbreeding coefficient, then the more inbred the founders, the more rapidly the population will be affected (Frankham *et al*., 2010). Based on the current N_e of 41.14, the increase in inbreeding rate to the next generation will be 0.012 ($1/(2N_e)$). This will increase the mean inbreeding to 0.0609 in one more generation; in the order of a 25% increase. It must be noted that inbreeding depression is commonly found in zoo populations across many taxa, and is therefore not unique to the Asiatic Lion (Boakes *et al*., 2007). Various characteristics are used as indicators of inbreeding depression, such as, juvenile mortality, birth size, litter size, reproductive rates, longevity and hereditary defects (Laikre, 1999). Some examples of taxa which have been assessed for the effects of inbreeding within captivity are ruffed lemurs (*Varecia variegata*), which displayed a reduced litter survival in offspring with a higher inbreeding coefficient (Noble *et al.,* 1990); wolves (*Canis lupus*), where hereditary blindness has been recorded in inbred lineages along with reduced litter sizes and longevity (Laikre, 1999); and cheetahs (*Acinonyx*

jubatus), which suffer from increased juvenile mortality in captivity compared to wild populations (Wielebnowski, 1996).

Inbreeding depression has also been observed in wild populations, normally associated with a reduction of numbers to low levels (Keller and Waller, 2002), which demonstrates that this issue is not unique to captive populations. However, it is harder to ascertain pedigrees for these wild populations to assess the levels of relatedness within the population (Liberg *et al.*, 2005), although as genetic tools become more abundant, and cheaper, relationships can be more readily established through field testing (Keller and Waller, 2002). Whilst inbreeding is generally naturally avoided in nature, when it does occur, there is a suggestion that the cost of inbreeding within these wild populations may be higher than within captive populations (Crnokrak and Roff, 1999). The authors suggest that this can be attributed to the environmental conditions the wild populations are exposed to, however, they do not go as far as to assign specifics to this assumption (Crnokrak and Roff, 1999).

Gene diversity is shown to be reducing by around 2.45% in every generation of the captive population. There is evidence that the representation, contribution and allele retention of certain founders results in the unbalanced representation of the GD, as the GD captured from some founders is prevalent across the living population, and others with much less frequency (Table 6). At this current rate of loss of gene diversity within this captive population, the goal of maintaining 90% of source variation for at least 100 years, cannot be met.

The cub mortality levels of around 70% in the period between 2007 and 2009 (and the total population mortality of over 50%) can be compared to a mortality rate of around 40% observed in Sakkarbaug zoo in the mid 1990's (Ashraf *et al*., 1993), and a recent study which showed 43% mortality in the Gir Forest population (Banerjee and Jhala, 2012). Cub

deaths in the wild are predominantly caused by starvation, abandonment and attacks by adult males in hierarchy battle. In captivity, where there is plentiful food and hand-rearing or fostering programmes in operation there should be a marked increase in survival rates (Crnokrak and Roff, 1999). This is not apparent in this population, and it has been shown that many species (ranging from rats (genus: *Rattus*) to giraffes (genus: *Giraffa*)) have high levels of infant mortality in captive populations, and these levels increase significantly in many species when there is evidence of inbreeding amongst the parental population (O'Brien *et al*., 1985, Ralls *et al*., 1979), suggesting that inbreeding may be a cause of these high mortality rates. A recent review of the mortality rates of Asiatic Lion within the EEP between 2000 and 2014 showed a total mortality rate of 73% (81 individuals) for cubs in the 'neonate' category (less than 1 month old) during the study period (Metz *et al.*, 2017). Stillbirths (15%), congenital defects (5%) and infectious disease (9%) account for 23 of the deaths, with 29 (36%) and 28 (35%) recorded as lack of care and trauma respectively.

It is imperative that genetic viability and compatibility should be considered carefully before arranging a mating between any dyads within the current population. Whilst the costs and ethical issues involved with transporting animals over long distances make it more appealing to arrange an exchange or transfer with a relatively local zoo, the long-term viability of the offspring, and therefore the captive population as a whole, must be considered. Whilst these lengthier transfers may have elevated costs and health implications, the longer term survival of any offspring produced in the partnership must make the process more attractive and financially advantageous to the establishments as they will benefit from the long term survival of a genetically viable population (Barnett *et al*., 2006b, Dubach *et al*., 2005).

5. Conclusion

The captured genetic variation is not comparable to captive Indian populations at the microsatellite level; this is not necessarily indicative of a complete lack of variation within the EEP population. However, it is of concern that variation was observed at these markers in the comparable captive population. Whilst the purpose of this particular research was to directly compare the variation between the two populations (Indian Captive and EEP), future research could increase the number of microsatellite loci selected to ascertain the depth of this homogeneity in the EEP population.

Other future research could also focus on two main areas: initially to attempt to locate variation in other areas of the genome, for example, using SNP's (Single Nucleotide Polymorphisms) or further microsatellite markers, and secondly to establish whether individuals from other populations may possess some variation which may be beneficial if introduced to the EEP population. However, it is imperative to avoid the possibility of introducing hybrid animals into this population, and the method of detection of hybrids must be agreed.

Positively, there have been species which have been successfully captive-bred from seemingly unrecoverable bottlenecks or the brink of extinction and hence, critically low levels of genetic variation (Spix McCaw (*Cyanopsitta spixii*), Przewalski's horse (*Equus przewalskii*), northern elephant seal (*Mirounga angustirostris*), Père David's deer (*Elaphurus davidianus*), Speke's Gazelle (*Gazella spekei*) (O'Brien *et al*., 1985, Frankham *et al*., 2010). However, it is vital that information provided by genetic research, combined with the studbook data and full use of software such as PMx, is utilised to ensure the best plan is made for the long-term survival of the captive species of the Asiatic Lion in the EEP.

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Appendix 1. Microsatellite primer sequences

All primers sourced from Gaur *et al*. (2006) and Singh *et al*. (2002).

Locus ID	Forward Primer Sequence (5′ to 3′)	Reverse Primer Sequence (5′ to 3′)	Expected product size (base pairs)	Fluorescent tag (5′ forward primer)
Ple23	GCTGCTCAAACAGGCTTCAC	CGCACACATCCGCTTCTACT	176-186	VIC
Ple24	GCTTCATGACTGAGCGTGAG	AACCACAGGCACTTCCTGAC	190-232	NED
Ple51	TCTCTCTCTGCTCCTCCCAG	CCCTAGCATCCTGCTCAGTC	174-187	FAM
Ple55	AGAGAGGGAACAGAGAGTG	CAGGTGTGGCTCCTTAAAC	148-163	PET
Ple57	CAGAGTGCGAGTGTGGACAT	CATGGAAATGACTTGGGGAC	128-156	FAM
Ple21	TCTCTGTGCCTCCGTTTCTT	GATGTGGGGCTTGAACTCAT	222-232	FAM
Ple34	ACCACACACATATCCGCATC	CCGATCCTTGAAGATTTTGC	118-124	PET
Ple53	GGATGTGAACTGGTGCAAAG	CGAGTGGTACTGCTGAGTCTG	117-129	NED
Ple58	TGCCACTGATGAGTCAAGTA	GTCCAAGATTCAGTGATCCA	221-227	PET
Ple62	CCCTCTCCCTGGTCACAC	GCCAACTGAGTTTGAGTCCC	155-169	NED
Ple65	GGAGCGAAACACGAAAACAG	CAGGAGCCTCATGCAGAGAT	110-120	VIC
Ple251	AGCTCTGGAAGGTCCTCATTC	CCCACTCATGCGTACACG	153-161	VIC

Appendix 2. mtDNA primer sequences

D-Loop primer sequences

(Shankaranarayanan and Singh, 1998)

Forward sequence: 5’ GCA TCT GGT TCT TAC TTC AGG 3’ (21 bases)
Reverse sequence: 5’ ATT TTC AGT GTC TTG CTT TT 3’ (20 bases)

Cytochrome *b* primer sequences

(Bertola *et al*., 2011)

Forward sequence: 5’ CGT TGT ACT TCA ACT ATA AGA ACT T 3’ (25 bases)
Reverse sequence: 5’ ATG GGA TTG CTG ATA GGA GAT TAG 3’ (24 bases)

Appendix 3. Summary of numbers of repeated PCRs in founder samples

First number represents the number of times a genotype was generated and the second is the number of PCRs carried out. Samples 1, 2, 7 and 8 are bone or skin samples; the rest are tissue. Grayscale represents no successful amplifications.

		Marker (Ple)											
		23	24	51	55	57	21	34	53	58	62	65	251
Individual	1	5/6	7/7	7/7	6/7	5/6	4/6	5/9	6/8	2/7	6/6	7/7	7/9
	2	3/7	7/7	7/8	3/6	6/6	0/9	5/8	5/8	0/9	5/8	6/6	7/9
	3	1/2	2/2	1/2	1/2	2/3	2/2	1/3	3/3	1/2	2/2	2/2	1/2
	4	1/2	1/2	1/1	1/1	1/2	3/3	1/2	2/2	1/2	1/3	2/2	1/2
	5	2/2	1/1	2/2	1/1	1/1	2/2	1/2	1/1	2/2	2/2	1/2	1/1
	6	1/2	1/1	2/2	2/2	1/2	2/2	0/2	2/2	1/3	1/3	1/2	1/2
	7	6/7	6/8	7/7	6/6	5/6	3/7	6/7	6/6	1/7	7/7	6/7	7/7
	8	4/6	7/7	5/7	4/8	2/7	2/6	0/9	3/6	1/8	6/6	2/7	6/8
	9	2/3	2/2	2/2	3/3	1/2	3/3	1/3	2/2	2/3	1/3	2/2	2/2

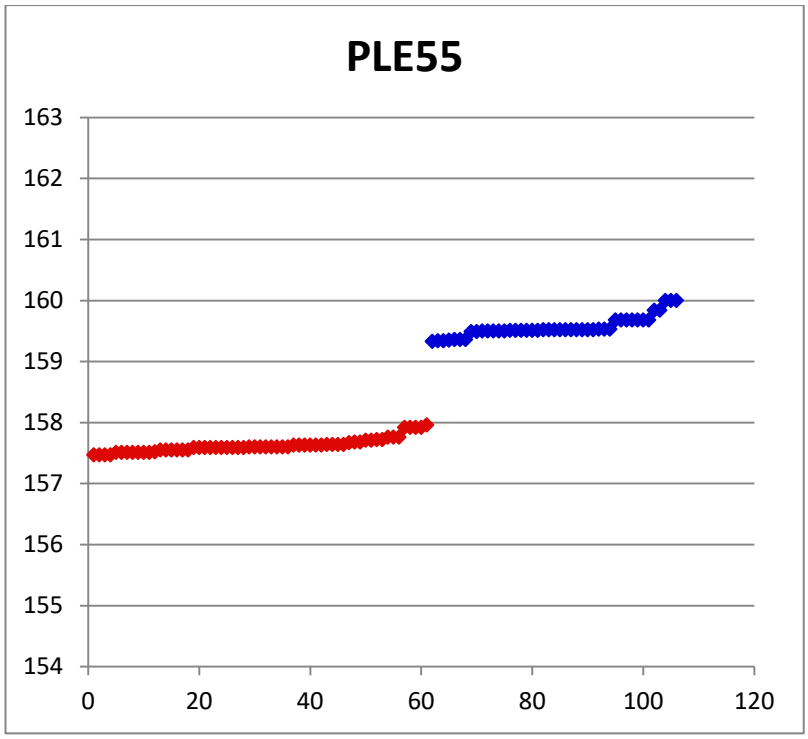
Appendix 4. Microsatellite Fragment sizes

Flexibin Results:

Marker (Ple)	Alleles designated*	Mean length	Standard Deviation	Shortest fragment	Longest fragment
23	1	179.82	0.365	179.32	180.66
24	1	192.02	0.156	191.74	192.97
	2	194.72	0.174	194.46	194.96
51	1	178.64	0.114	178.33	178.91
55	1	157.62	0.116	157.47	157.96
	2	159.56	0.166	159.33	160.00
57	1	149.36	0.146	149.08	149.64
	2	151.41	0.113	151.21	151.68
21	1	231.58	0.307	230.94	232.37
34	1	124.33	0.077	124.16	124.54
53	1	125.87	0.112	125.62	126.11
58	1	228.27	0.113	228.07	228.53
62	1	167.46	0.172	167.15	167.82
65	1	119.04	0.121	118.71	119.31
251	1	155.06	0.130	154.82	155.56

* Alleles designated as ‘1’ or ‘2’ by software with no inference to number of repeats observed at each locus

Example Flexibin output (Locus Ple55):



Microsatellite fragment sizes for founder samples (1-9)

	Mean fragment length allele 1 (bp) (*Standard Deviation*) **Mean fragment length allele 2 (bp)** (*Standard Deviation*)											
	Ple marker											
ID	23	24	51	55	57	21	34	53	58	62	65	251
1	**179.83** *(0.095)*, **179.83** *(0.095)*	**192.02** *(0.081)*, **192.02** *(0.081)*	**178.60** (0.116), **178.60** *(0.116)*	**157.57** (0.069), **159.44** *(0.080)*	**149.26** *(0.152)*, **149.26** (0.152)	**231.35** *(0.230)*, **231.35** *(0.230)*	**124.29** *(0.042)*, **124.29** *(0.042)*	**125.88** *(0.128)*, **125.88** *(0.128)*	**228.50** *(0.064)*, **228.50** *(0.064)*	**167.44** *(0.143)*, **167.44** *(0.143)*	**118.97** *(0.077)*, **118.97** *(0.077)*	**154.99** *(0.095)*, **154.99** *(0.095)*
2	**179.49** *(0.121)*, **179.49** *(0.121)*	**191.97** *(0.102)*, **191.97** *(0.102)*	**178.64** *(0.111)*, **178.64** *(0.111)*	**157.65** *(0.192)*, **159.60** *(0.164)*	**149.43** *(0.107)*, **149.43** *(0.107)*	X	**124.34** *(0.091)*, **124.34** *(0.091)*	**125.84** *(0.094)*, **125.84** *(0.094)*	X	**167.59** *(0.243)*, **167.59** *(0.243)*	**118.98** *(0.089)*, **118.98** *(0.089)*	**155.11** *(0.109)*, **155.11** *(0.109)*
3	**179.53** *(N/A)*, **179.53** *(N/A)*	**192.04** *(0.021)*, **192.04** *(0.021)*	**178.58** *(N/A)*, **178.58** *(N/A)*	**157.64** *(N/A)*, **159.53** *(N/A)*	**149.35** *(0.163)*, **149.35** *(0.163)*	**231.43** *(0.276)*, **231.43** *(0.276)*	**124.27** *(0.027)*, **124.27** *(0.027)*	**125.93** *(0.111)*, **125.93** *(0.111)*	**228.07** *(N/A)*, **228.07** *(N/A)*	**167.43** *(0.149)*, **167.43** *(0.149)*	**119.09** *(0.028)*, **119.09** *(0.028)*	**155.08** *(N/A)*, **155.08** *(N/A)*
4	**179.61** *(N/A)*, **179.61** *(N/A)*	**191.90** *(N/A)*, **191.90** *(N/A)*	**178.81** *(N/A)*, **178.81** *(N/A)*	**157.72** *(N/A)*, **159.68** *(N/A)*	**151.51** *(0.240)*, **151.51** *(0.240)*	**231.71** *(0.387)*, **231.71** *(0.387)*	**124.30** *(0.014)*, **124.30** *(0.014)*	**125.89** *(0.103)*, **125.89** *(0.103)*	**228.23** *(N/A)*, **228.23** *(N/A)*	**167.72** *(N/A)*, **167.72** *(N/A)*	**119.07** *(0.057)*, **119.07** *(0.057)*	**155.23** *(N/A)*, **155.23** *(N/A)*
5	**180.08** *(0.551)*, **180.08** *(0.551)*	**192.07** *(N/A)*, **192.07** *(N/A)*	**178.68** *(0.226)*, **178.68** *(0.226)*	**157.59** *(0.000)*, **159.52** *(0.113)*	**151.38** *(0.021)*, **151.38** *(0.021)*	**231.73** *(0.515)*, **231.73** *(0.515)*	**124.36** *(0.134)*, **124.36** *(0.134)*	**126.00** *(0.092)*, **126.00** *(0.092)*	**228.35** *(0.087)*, **228.35** *(0.087)*	**167.29** *(0.156)*, **167.29** *(0.156)*	**119.03** *(N/A)*, **119.03** *(N/A)*	**155.15** *(N/A)*, **155.15** *(N/A)*
6	**179.97** *(0.058)*, **179.97** *(0.058)*	**191.86** *(N/A)*, **191.86** *(N/A)*	**178.82** *(0.134)*, **178.82** *(0.134)*	**157.65** *(0.085)*, **157.65** *(0.085)*	**149.27** *(0.012)*, **151.31** *(0.087)*	**231.48** *(0.318)*, **231.48** *(0.318)*	X	**125.88** *(0.214)*, **125.88** *(0.214)*	**228.22** *(N/A)*, **228.22** *(N/A)*	**167.62** *(N/A)*, **167.62** *(N/A)*	**118.91** *(N/A)*, **118.91** *(N/A)*	**155.14** *(N/A)*, **155.14** *(N/A)*
7	**179.71** *(0.204)*, **179.71** *(0.204)*	**191.99** *(0.071)*, **191.99** *(0.071)*	**178.62** *(0.093)*, **178.62** *(0.093)*	**157.60** *(0.080)*, **157.60** *(0.080)*	**151.36** *(0.075)*, **151.36** *(0.075)*	**231.50** *(0.366)*, **231.50** *(0.366)*	**124.36** *(0.070)*, **124.36** *(0.070)*	**125.85** *(0.115)*, **125.85** *(0.115)*	**228.20** *(0.050)*, **228.20** *(0.050)*	**167.38** *(0.120)*, **167.38** *(0.120)*	**119.07** *(0.119)*, **119.07** *(0.119)*	**154.97** *(0.076)*, **154.97** *(0.076)*
8	**179.49** *(0.111)*, **179.49** *(0.111)*	**192.02** *(0.103)*, **194.72** *(0.170)*	**178.57** *(0.080)*, **178.57** **(0.080)**	**157.55** *(0.061)*, **159.43** *(0.093)*	**149.32** *(0.092)*, **151.41** *(0.073)*	**231.48** *(0.164)*, **231.48** *(0.164)*	X	**125.78** *(0.067)*, **125.78** *(0.067)*	**228.28** *(N/A)*, **228.28** *(N/A)*	**167.54** *(0.186)*, **167.54** *(0.186)*	**119.12** *(0.101)*, **119.12** *(0.101)*	**155.18** *(0.175)*, **155.18** *(0.175)*
9	**179.96** *(0.884)*, **179.96** *(0.884)*	**191.93** *(0.205)*, **191.93** *(0.205)*	**178.66** *(0.071)*, **178.66** *(0.071)*	**157.80** *(0.186)*, **159.76** *(0.211)*	**149.46** *(0.000)*, **151.52** *(0.007)*	**231.57** *(0.113)*, **231.57** *(0.113)*	**124.34** *(0.064)*, **124.34** *(0.064)*	**125.88** *(0.115)*, **125.88** *(0.115)*	**228.38** *(0.100)*, **228.38** *(0.100)*	**167.57** *(N/A)*, **167.57** *(N/A)*	**119.11** *(0.099)*, **119.11** *(0.099)*	**155.15** *(0.127)*, **155.15** *(0.127)*

X– no genotype was established for this individual at this loci.

N/A - only one result was obtained so no standard deviation calculated

Microsatellite fragment sizes for contemporary samples (1-7)

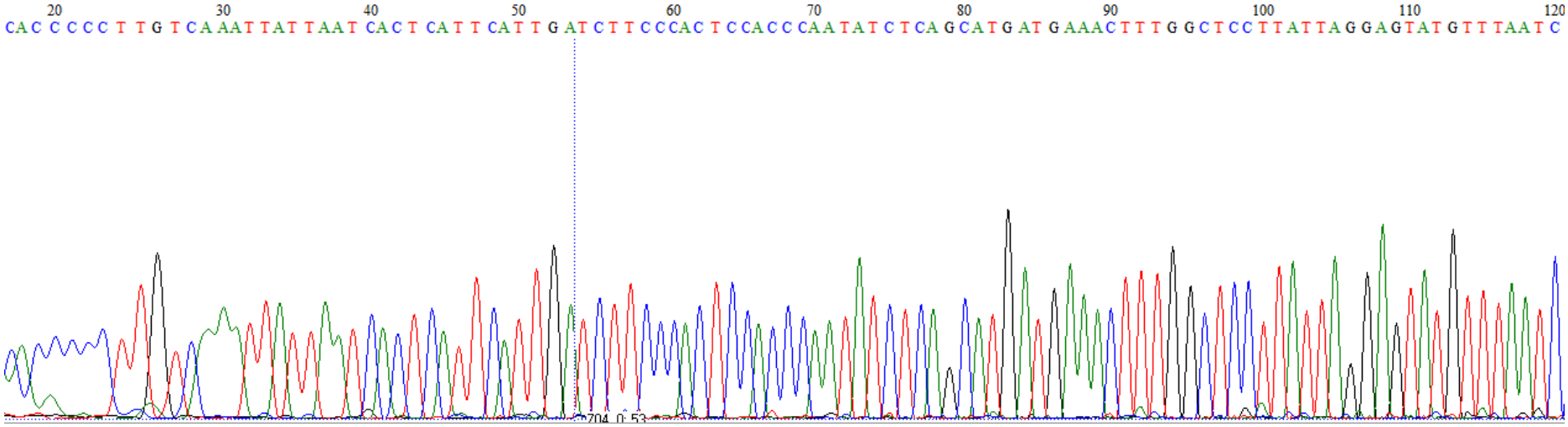
	Mean fragment length allele 1 (bp) (*Standard Deviation*) **Mean fragment length allele 2 (bp)** (*Standard Deviation*)											
	Ple marker											
ID	23	24	51	55	57	21	34	53	58	62	65	251
1	**180.47** *(N/A),* **180.47** *(N/A)*	X	**178.78** *(N/A),* **178.78** *(N/A)*	**159.77** *(0.332),* **159.77** *(0.332)*	**149.46** *(0.000),* **151.52** *(0.006)*	**231.63** *(0.157),* **231.63** *(0.157)*	**124.31** *(0.064),* **124.31** *(0.064)*	**125.94** *(0.191),* **125.94** *(0.191)*	**228.37** *(0.099),* **228.37** *(0.099)*	**167.44** *(N/A),* **167.44** *(N/A)*	**119.01** *(N/A),* **119.01** *(N/A)*	**155.15** *(N/A),* **155.15** *(N/A)*
2	**180.28** *(N/A),* **180.28** *(N/A)*	**192.14** *(0.057),* **192.14** *(0.057)*	**178.61** *(N/A),* **178.61** *(N/A)*	**159.57** *(0.095),* **159.57** *(0.095)*	**151.44** *(0.113),* **151.44** *(0.113)*	**231.73** *(0.586),* **231.73** *(0.586)*	**124.31** *(0.141),* **124.31** *(0.141)*	**126.00** *(0.099),* **126.00** *(0.099)*	**228.28** *(0.068),* **228.28** *(0.068)*	**167.46** *(N/A),* **167.46** *(N/A)*	**118.93** *(0.226),* **118.93** *(0.226)*	**155.03** *(0.170),* **155.03** *(0.170)*
3	**180.56** *(N/A),* **180.56** *(N/A)*	**192.16** *(0.028),* **192.16** *(0.028)*	**178.78** *(0.071),* **178.78** *(0.071)*	**159.60** ***(0.113),*** **159.60** *(0.113)*	**149.28** *(N/A),* **151.35** *(N/A)*	**231.59** *(0.156),* **231.59** *(0.156)*	**124.31** *(0.057),* **124.31** *(0.057)*	**125.87** *(0.000),* **125.87** *(0.000)*	**228.18** *(0.028),* **228.18** *(0.028)*	**167.29** *(N/A),* **167.29** *(N/A)*	**119.15** *(N/A),* **119.15** *(N/A)*	**155.11** *(0.297),* **155.11** *(0.297)*
4	**180.50** *(N/A),* **180.50** *(N/A)*	**191.76** *(N/A),* **191.76** *(N/A)*	**178.74** *(0.090),* **178.74** *(0.090)*	**157.68** *(0.106),* **159.60** *(0.113)*	**149.58** *(0.098),* **151.50** *(0.160)*	**231.70** *(0.103),* **231.70** *(0.103)*	**124.35** *(0.010),* **124.35** *(0.010)*	**125.87** *(0.007),* **125.87** *(0.007)*	**228.23** *(0.028),* **228.23** *(0.028)*	**167.43** *(N/A),* **167.43** *(N/A)*	**119.19** *(N/A),* **119.19** *(N/A)*	**155.15** *(N/A),* **155.15** *(N/A)*
5	**180.66** *(N/A),* **180.66** *(N/A)*	**191.82** *(N/A),* **191.82** *(N/A)*	**178.58** *(0.085),* **178.58** *(0.085)*	**157.66** *(0.092),* **159.44** *(0.106)*	**149.37** *(0.127),* **151.44** *(0.113)*	**231.93** *(0.417),* **231.93** *(0.417)*	**124.49** *(0.042),* **124.49** *(0.042)*	**126.02** *(N/A),* **126.02** *(N/A)*	**228.22** *(0.028),* **228.22** *(0.028)*	**167.38** *(0.198),* **167.38** *(0.198)*	**119.26** *(N/A),* **119.26** *(N/A)*	**155.14** *(N/A),* **155.14** *(N/A)*
6	**180.02** *(0.445),* **180.02** *(0.445)*	**192.22** *(N/A),* **192.22** *(N/A)*	**178.74** *(0.095),* **178.74** *(0.095)*	**157.78** *(0.198),* **157.78** *(0.198)*	**151.50** *(0.219),* **151.50** *(0.219)*	**231.61** *(0.282),* **231.61** *(0.282)*	**124.26** *(0.134),* **124.26** *(0.134)*	**125.81** *(0.040),* **125.81** *(0.040)*	**228.21** *(0.114),* **228.21** *(0.114)*	**167.35** *(0.014),* **167.35** *(0.014)*	**119.15** *(N/A),* **119.15** *(N/A)*	**155.03** *(0.184),* **155.03** *(0.184)*
7	**180.12** *(0.512),* **180.12** *(0.512)*	**192.58** *(0.559),* **192.58** *(0.559)*	**178.63** *(0.057),* **178.63** *(0.057)*	**157.57** *(0.028),* **159.52** *(0.007)*	**149.46** *(0.000),* **151.51** *(0.000)*	**231.71** *(0.368),* **231.71** *(0.368)*	**124.46** *(N/A),* **124.46** *(N/A)*	**125.85** *(0.062),* **125.85** *(0.062)*	**228.20** *(0.084),* **228.20** *(0.084)*	**167.39** *(0.028),* **167.39** *(0.028)*	**118.89** *(0.255),* **118.89** *(0.255)*	**155.06** *(N/A),* **155.06** *(N/A)*

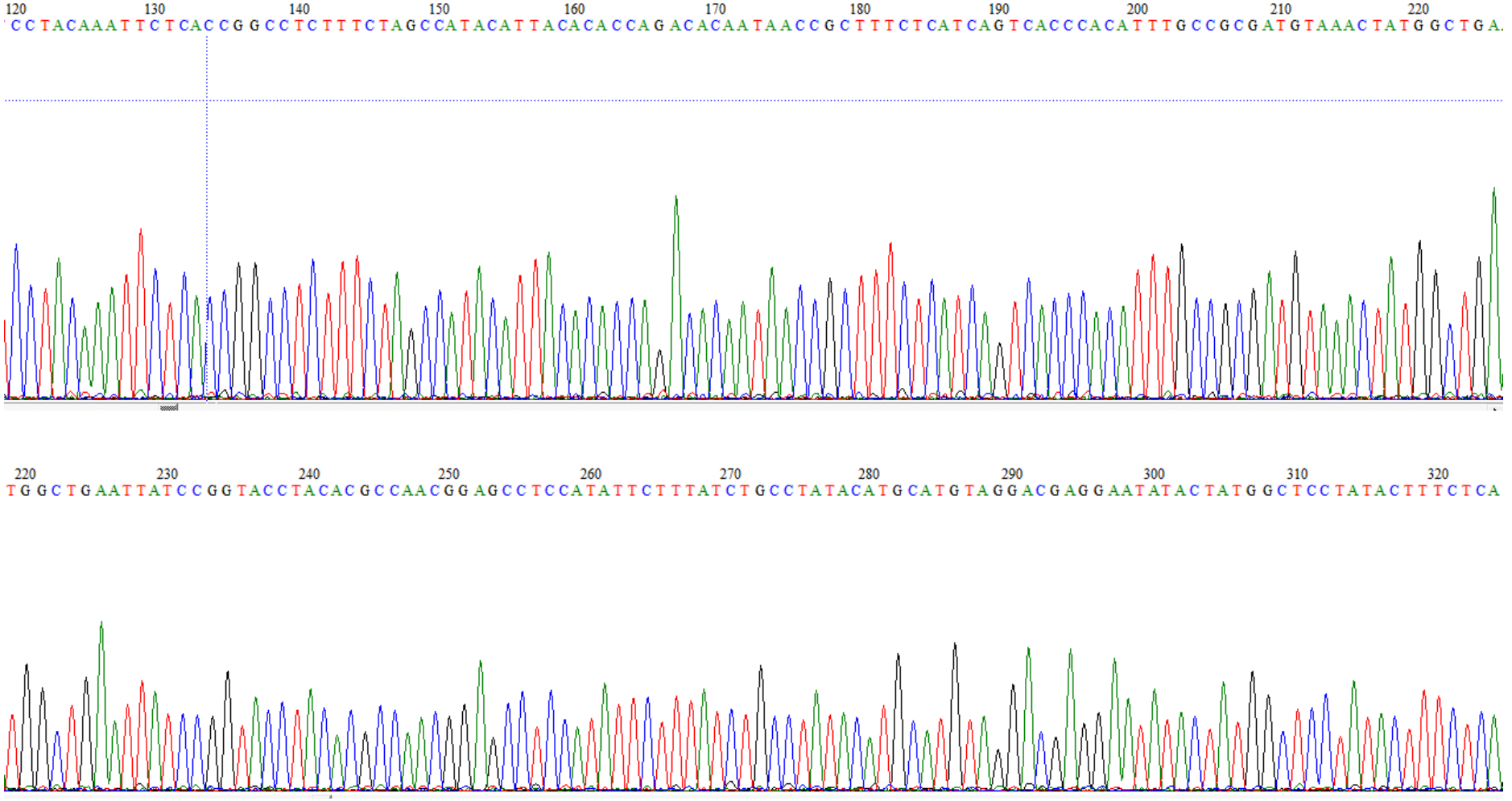
X– no genotype was established for this individual at this loci.

N/A - only one result was obtained so no standard deviation calculated

Appendix 5. Example DNA sequences

Cytochrome *b* Forward sequence: Individual 4




120 130 140 150 160 170 180 190 200 210 220
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220 230 240 250 260 270 280 290 300 310 320
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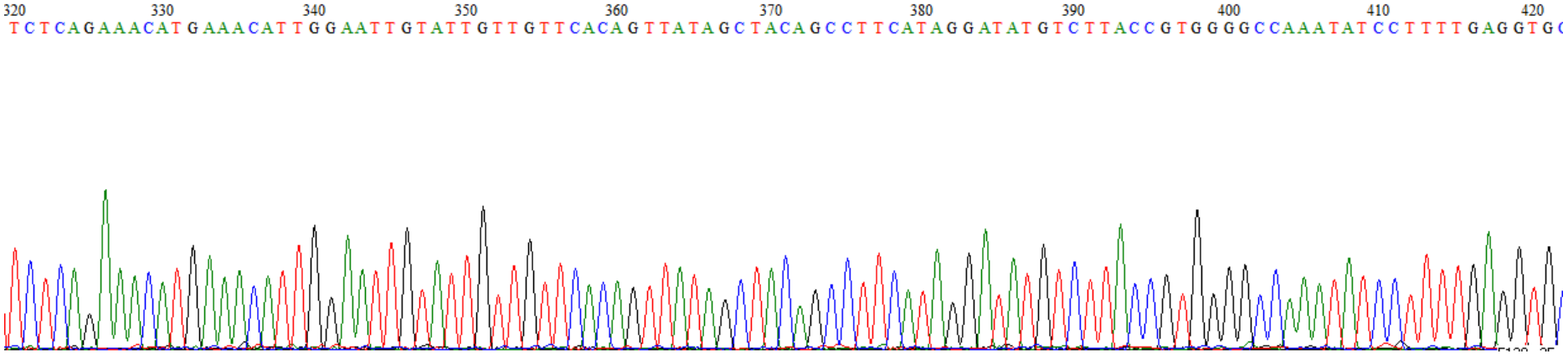
320 330 340 350 360 370 380 390 400 410 420
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420 430 440 450 460
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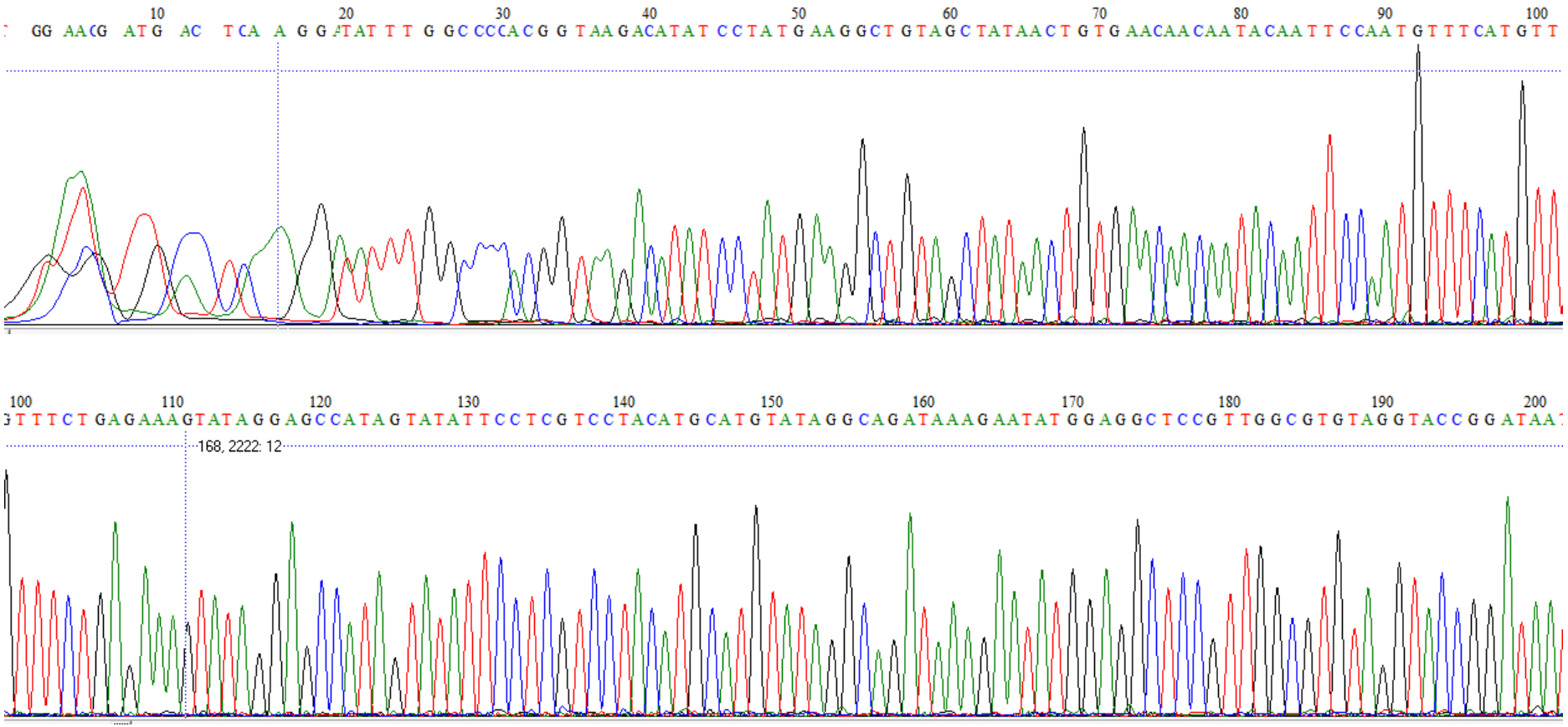
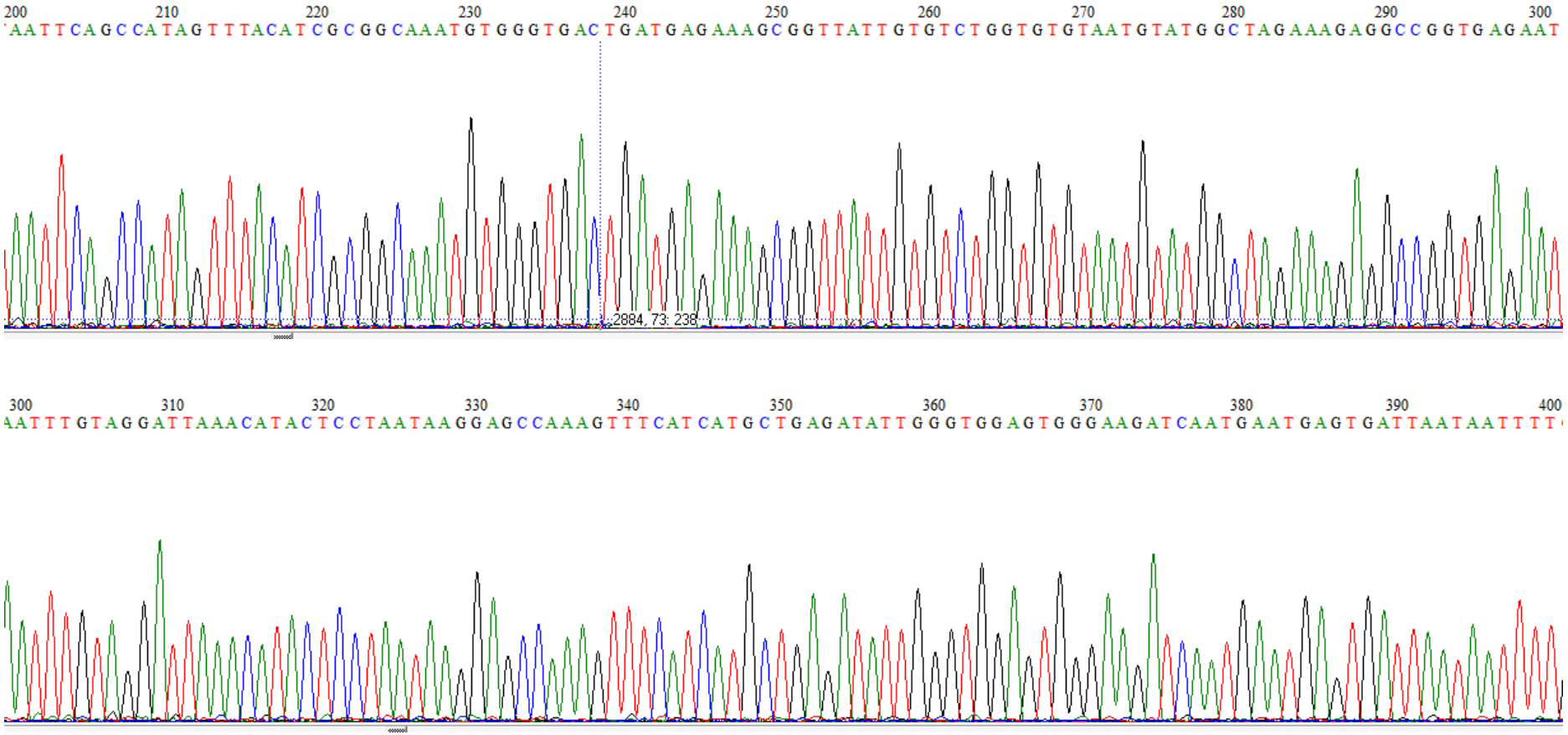
Cytochrome *b* Reverse sequence: Individual 4

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AATTCAGCCATAGTTTACATCGCGGCAAATGTGGGTGACTGATGAGAAAGCGGTTATTGTGTCTGGTGTGTAATGTATGGCTAGAAAGAGGCCGGTGAGAAT
2884, 73: 238
300 310 320 330 340 350 360 370 380 390 400
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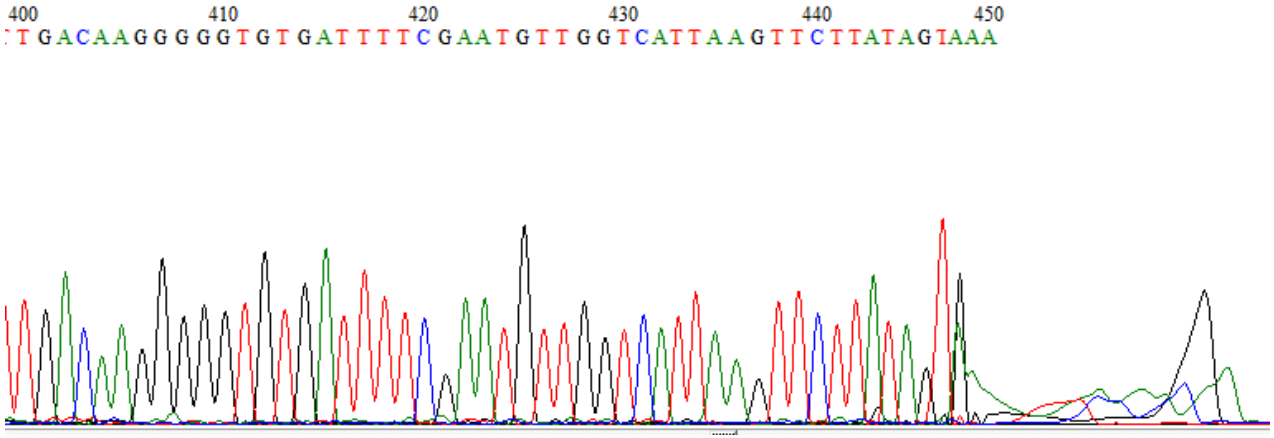
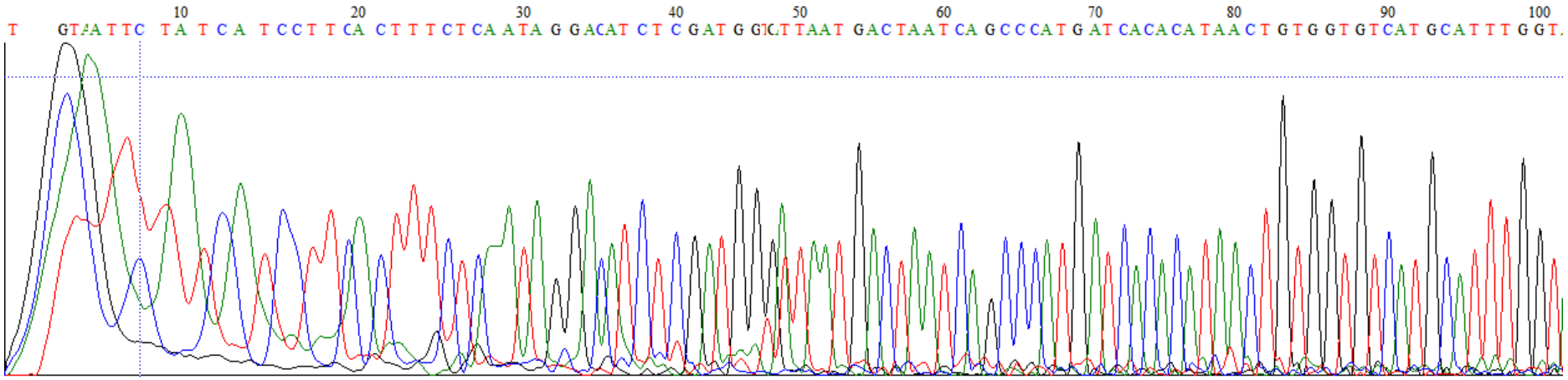
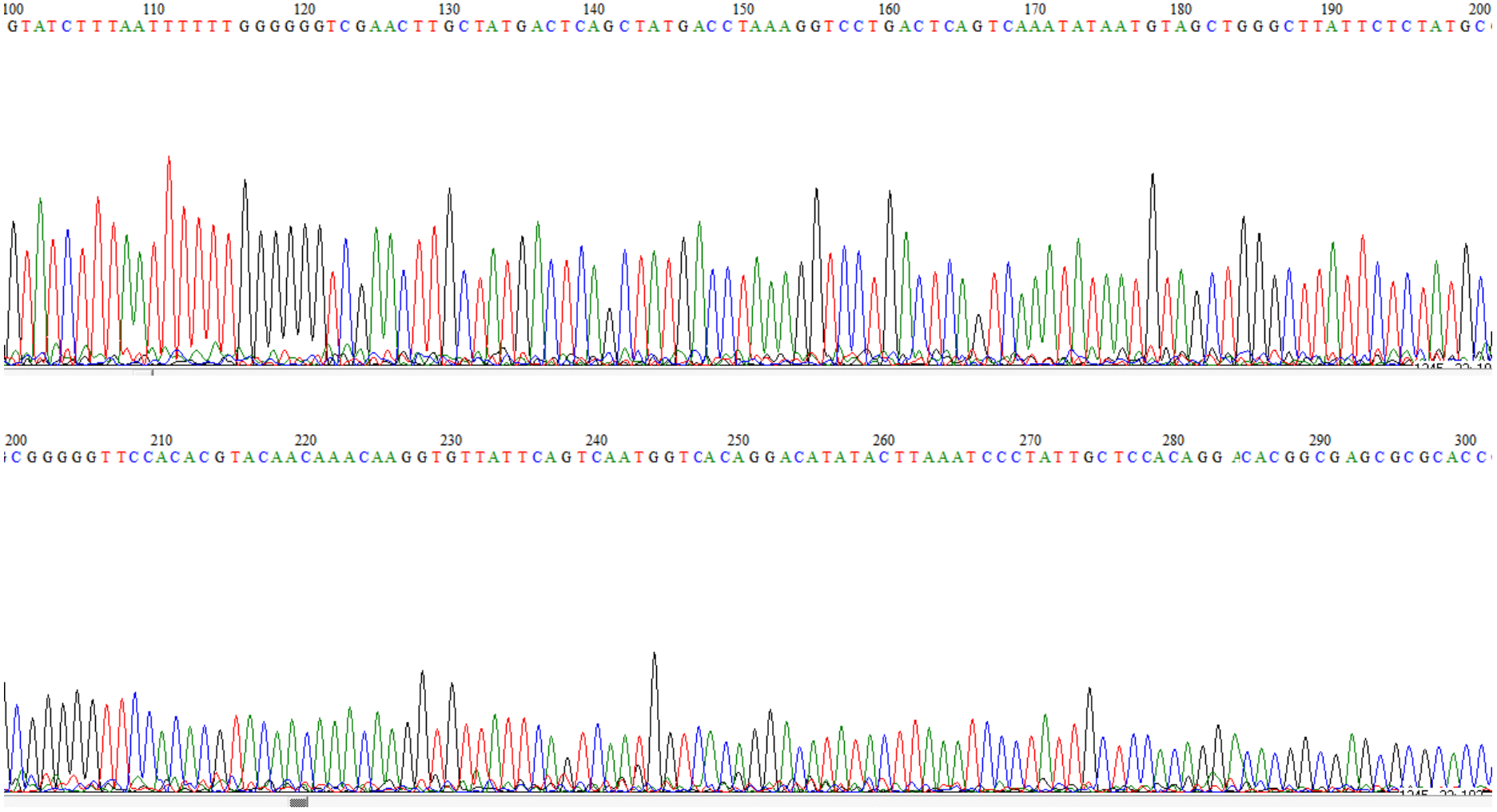

400
410
420
430
440
450
T G A C A A G G G G G T G T G A T T T T C G A A T G T T G G T C A T T A A G T T C T T A T A G T A A A

D-Loop Forward sequence: Individual 6

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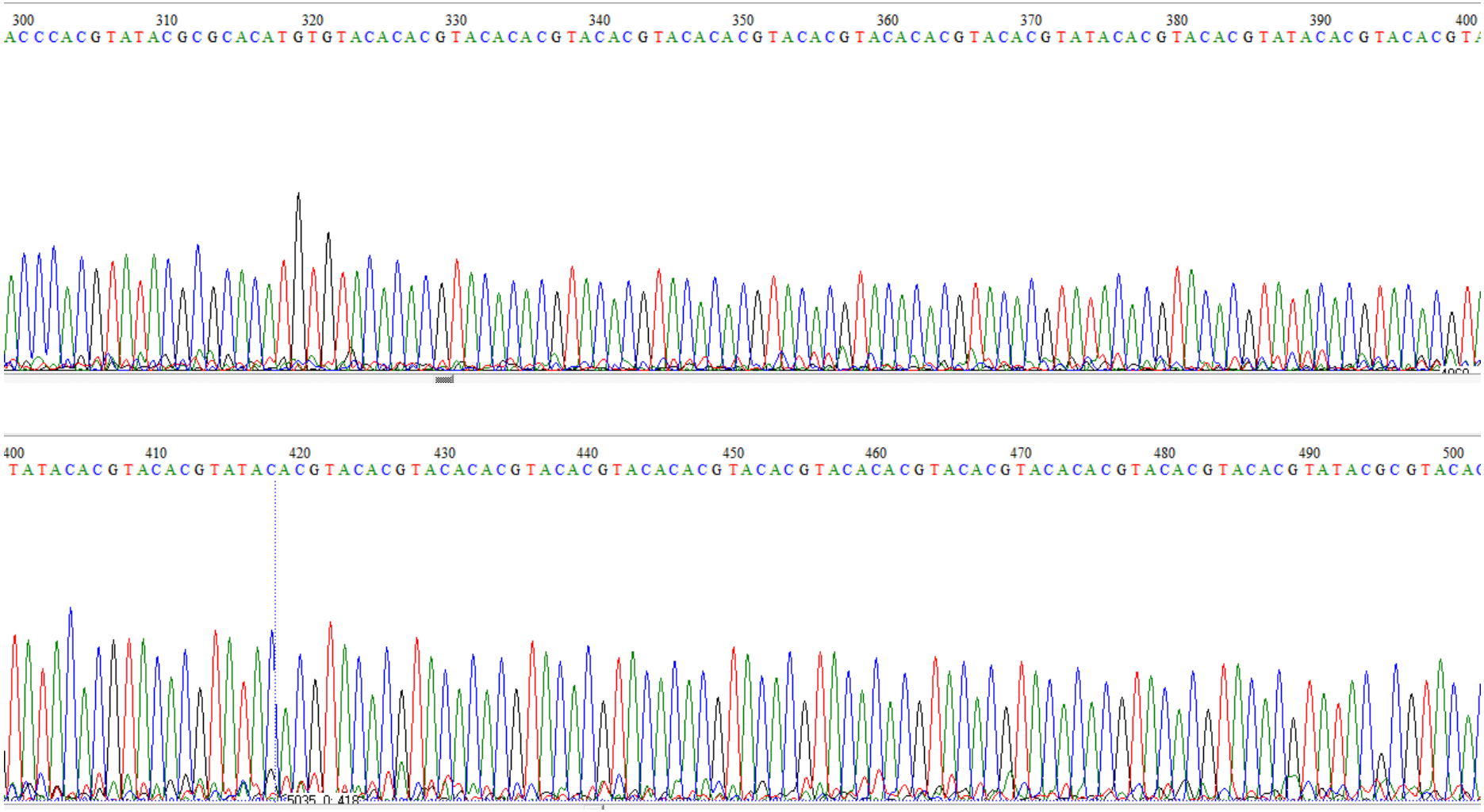
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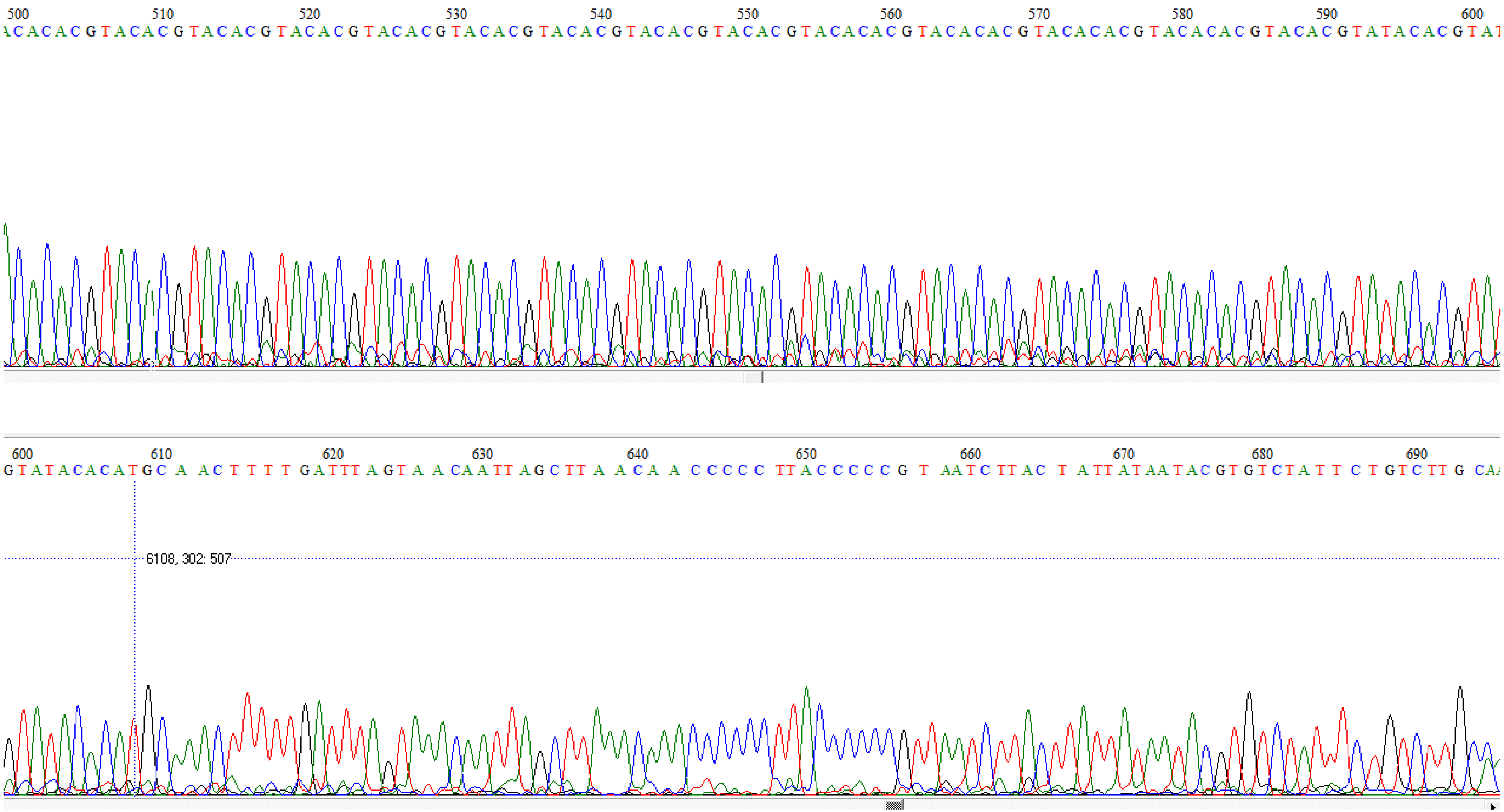
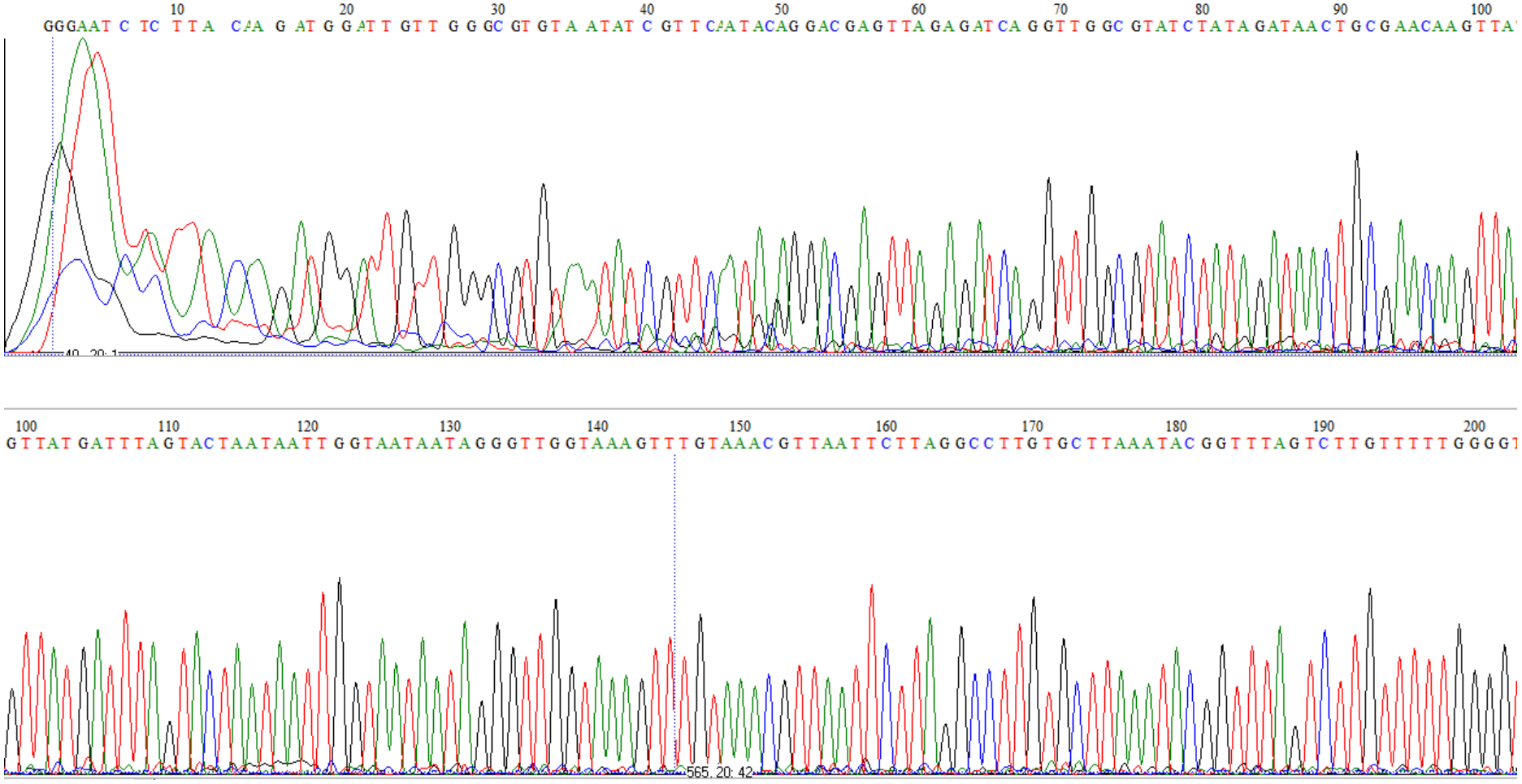
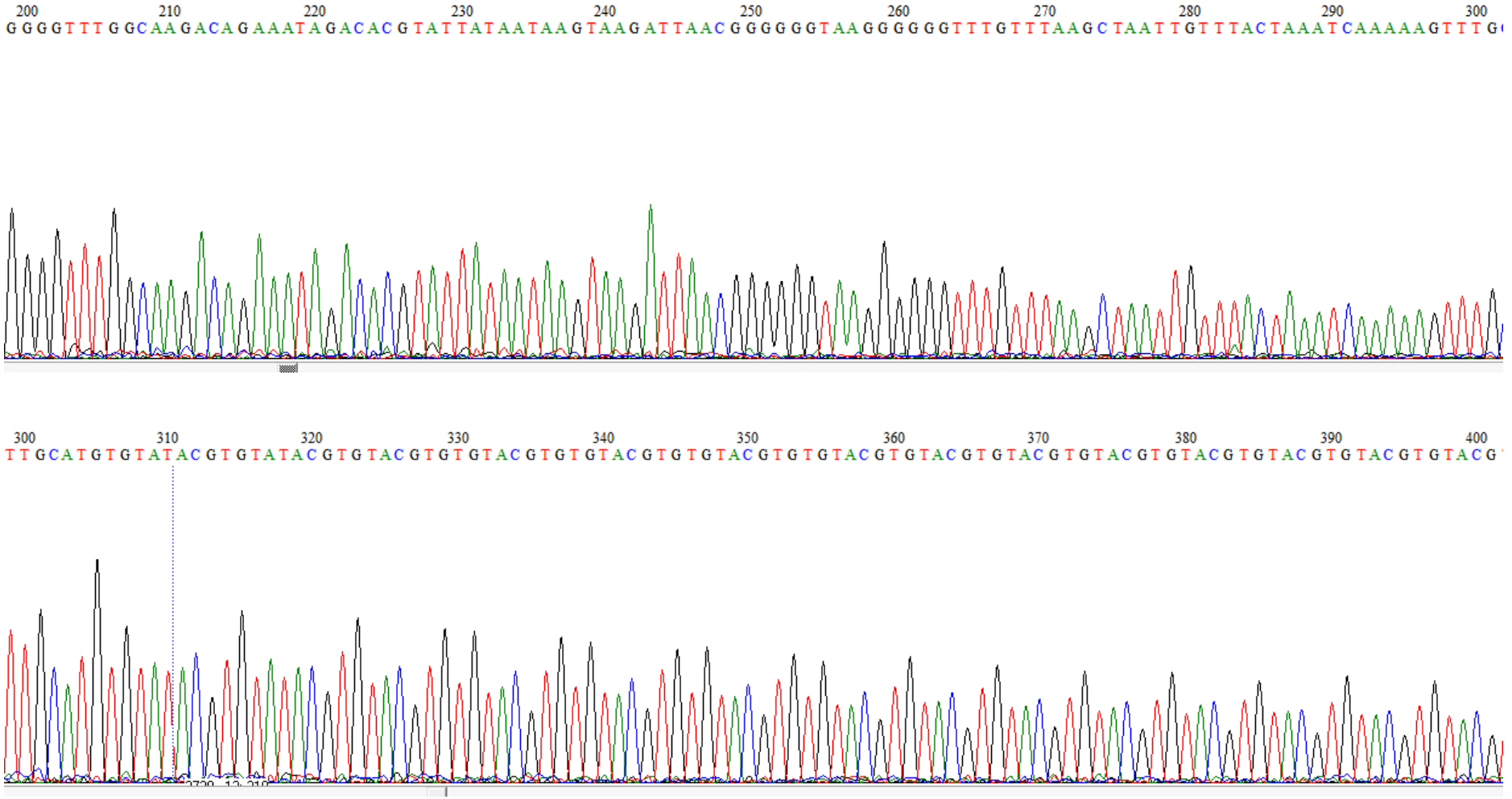


D-Loop Reverse sequence: Individual 6

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GGGGTTTGGCAAGACAGAAATAGACACGTATTATAATAAGTAAGATTAACGGGGGGGTAAGGGGGGGTTTGTTTAAGCTAATTGTTTACTAAATCAAAAAGTTTG
300 310 320 330 340 350 360 370 380 390 400
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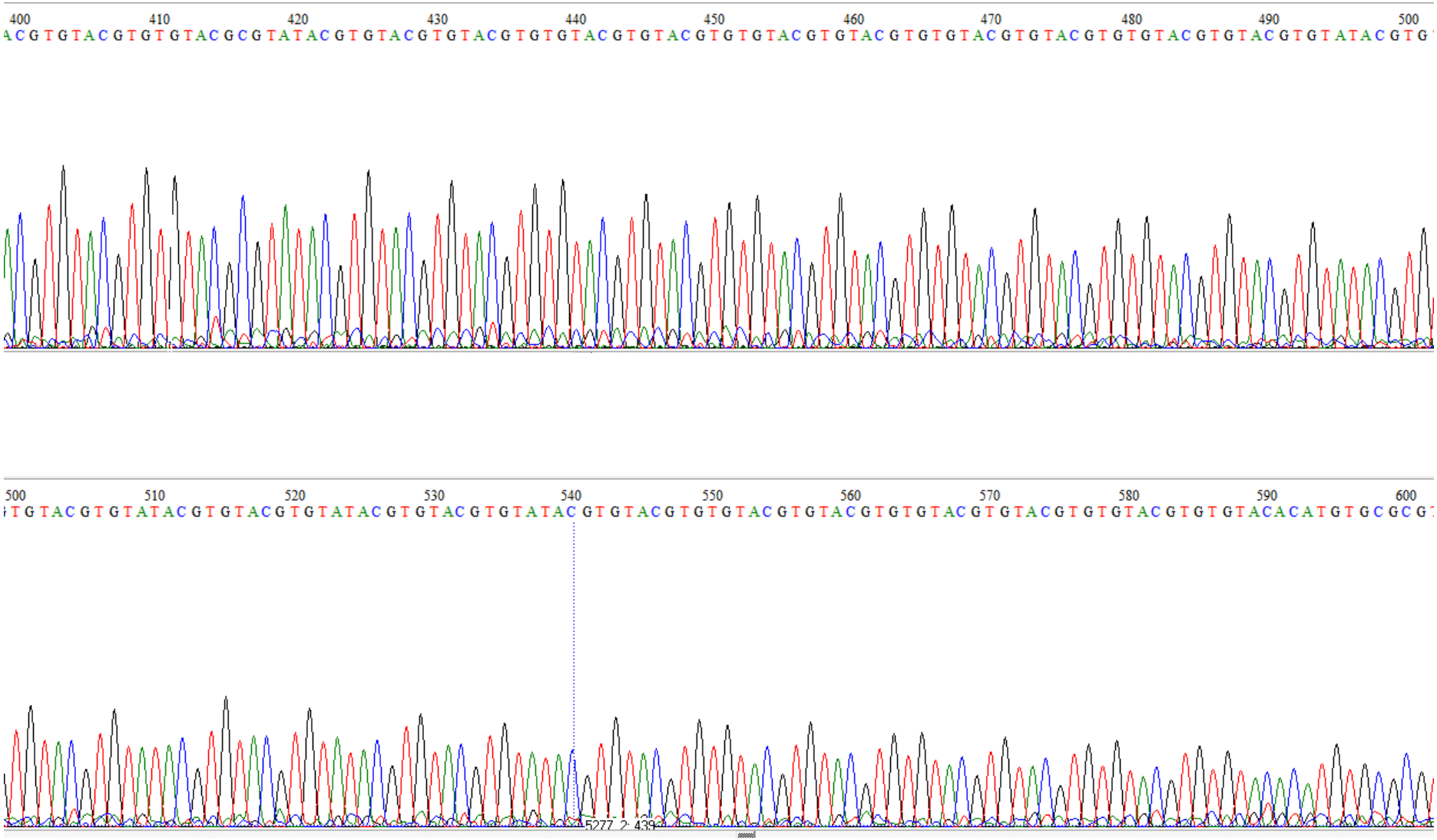
400 410 420 430 440 450 460 470 480 490 500
ACGTGTACGTGTGTACGCGTATACGTGTACGTGTACGTGTGTACGTGTACGTGTGTACGTGTACGTGTGTACGTGTACGTGTGTACGTGTACGTGTGTATACGTG
500 510 520 530 540 550 560 570 580 590 600
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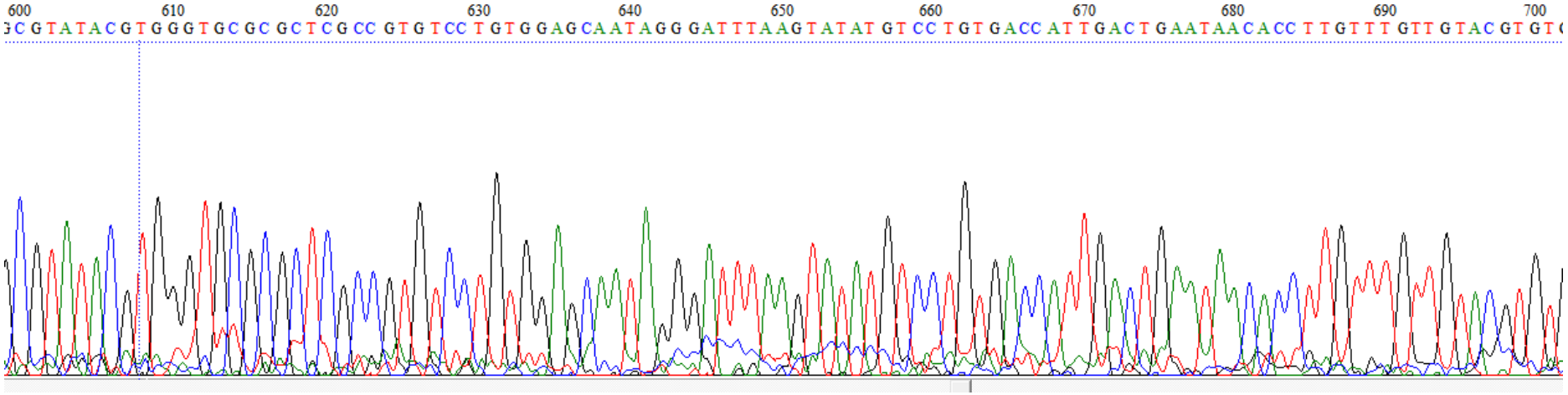
600 610 620 630 640 650 660 670 680 690 700
GCGTATACGTGGGTGCGCGCTCGCCGTGTCCTGTGGAGCAATAGGGATTTAAGTATATGTCCTGTGACCATTGACTGAATAACACCTTGTTTGTTGTACGTGTC

Glossary

Allele - Alternative versions of sections of DNA (coding or non-coding) found in a particular area of the genome. Diploid species have two alleles at each genetic locus.

Amplification - Technique which multiplies target copes of DNA strands so that they can be visualised.

Bottleneck - When a population is drastically reduced in numbers due to external factors (e.g. natural disaster, disease, predation, environmental threats).

Chromosome - A thread-like structure found in the cell nucleus which consists of a chain of DNA and associated proteins.

Demographic - Data which is statistically derived from a population (e.g. age, births, deaths, migration).

DNA - Deoxyribonucleic Acid. Molecule which carries the genetic code to programme the synthesis of proteins which allow living things to function, reproduce and develop.

DNA sequencing - The chemical process which allows a strand of DNA to be read 'base by base'.

Dyad - A mating pair

Founders - The individuals which are classed as the initial members of a population.

Founder Effect - The changes in genetic composition within and between populations as a result of the establishment of a new isolated group from a small number of founders.

Genotype - The genetic profile (designated alleles) at a single locus for an individual, or a combination of genetic profiles from multiple loci within an individual.

Genome - The complete set of DNA in an individual. There are 2 genomes (Nuclear and Mitochondrial). Each cell has one nuclear genome but thousands of mitochondrial genomes.

Hardy-Weinberg Equilibrium (HWE) - A hypothetical state where the allele frequencies within a population remain constant over generations. The criteria are not normally met in any population but calculations can be made to confirm that data is in HWE.

Heterozygote - When an individual possesses two different versions of an allele at a microsatellite locus, i.e. they have inherited different copies from each parent (visualised by two peaks at the locus).

$\mathbf{H_E}$ - Expected Heterozygosity. Once all known alleles within a population are known, the proportion of individuals which are expected to be heterozygous is calculated.

$\mathbf{H_o}$ - Observed heterozygosity. The actual proportion of individuals which are heterozygous.

Homozygote - When an individual possesses the same versions of an allele at a microsatellite locus, i.e. when they have inherited the same allele from both parents (visualised by a single peak at the locus).

Inbreeding -Where offspring are produced through mating between individuals who have descended from the same ancestors i.e. where individuals are closely related. The level of inbreeding is described using F-values.

Inter-population - Between populations.

Intra-population - Within populations.

Locus (pl:loci) – The genetic location on a chromosome.

Mean Kinship - The average level of relatedness to other members of the population.

Microsatellite - An area (locus) of DNA with tandem repeating sequences.

mtDNA - Mitochondrial DNA. Inherited solely from the maternal lineage. Occurs within the mitochondria in the cell and there are thousands of copies within each cell, but contains limited information in comparison to the nuclear genome.

Mutation - A genetic change in the genome of an individual. Can be a single nucleotide change or insertion or deletion of sections.

Nucleotide - A chemical compound consisting of a sugar, phosphate and 'base'. These compounds create the complex molecular structure of the DNA strands. The bases are Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). These bases pair in specific complimentary formations (A with T; C with G).

PCR – Polymerase Chain Reaction. Molecular technique which artificially replicates a targeted section of DNA through the process of thermal cycling, resulting in an increase of the targeted fragment (by several orders of magnitude).

Primers - A short artificially manufactured strand of DNA (normally about 20-25 nucleotides) which is designed to attach to a region of target DNA to allow *in-vitro* amplification.

Variability - An assessment of the number of different versions of the genetic or phenotypic features observed within a target population.