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Prifysgol De Cymru

The Effectiveness of Non-Invasive Sampling of Leopards (*Panthera pardus*) for Genetic Analysis at the Selati Game Reserve, South Africa

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Abstract

With increasing use of genetics for evidencing ecological understanding to a remarkable degree of accuracy, it has become increasingly imperative to gain biological samples by non-invasive means. In order to investigate the viability of non-invasive genetics, this study focused on three aims to compare sample types, implement an innovative non-invasive field method and test the efficiency of the field technique in obtaining genetic information.

African lion (*Panthera leo*) hair, faecal and tissue samples were collected in the Selati Game Reserve, South Africa and then tested for DNA quantity and quality upon extraction in the University of South Wales (USW) laboratory. An innovative non-invasive hair sampling system was applied to African leopards (*Panthera pardus pardus*) of the same study site whereby adhesive tape was strategically placed on baited trees throughout the reserve. These samples were then transferred to USW to test for genetic viability of the technique by application of Polymerase Chain Reaction (PCR) and statistical sequence match analysis.

This study identified hair samples to be the greatest source of DNA in terms of DNA concentration and purity, followed by tissue and faeces thus supporting sample choice for the proposed field methodology. The bait trap system was highly effective in obtaining hair samples from all sites where leopards were recorded as present. Genetic amplification occurred by use of *cytochrome b* universal primers, and although sequencing was unsuccessful in identifying leopard as the DNA origin, a significant species match was produced for African civet (similarity = 98%, E= 2e-146) that used that bait system. This study demonstrates how non-invasive sampling can be employed to provide genetic information on elusive species, but highlights how sample processing steps should be optimised for the study subject. Future research should consider quantifying genotyping errors and primer-specificity to ensure accurate results on target species.

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Abbreviations

%	Percent
‘	Minute(s)
‘’	Second(s)
®	Registered trademark
°	Degrees
°C	Degrees Celsius
µg	Micrograms
µl	Microliter(s)
3’	Three prime
5’	Five prime
AE	Elution buffer
AL	Animal lysis
ASL	Animal Stool Lysis
ATL	Animal Tissue Lysis
ATP6	*Adenosine triphosphate 6*
AW	Column Wash buffer
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO1	*Cytochrome c oxidase subunit I*
CTAB	Cetyltrimethyl ammonium bromide
Cytb	*Cytochrome b*
DET	Dimethyl sulfoxide; EDTA; Tris; Sodium chloride
DFED	Department of Finance and Economic Development
dH_20	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
E	Expected (value)
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FST	Fixation index
g	Gram(s)
GB	Gigabyte
h	hour
Ha	Hectare
HCl	Hydrochloric acid
Km	Kilometre
m	Metre
M	Molar
Mg	Microgram
$MgCl_2$	Magnesium Chloride
min	Minute
Ml	Microliter
Mm	Millilitre
mtDNA	Mitochondrial DNA
NaCl	Sodium Chloride
NaOH	Sodium hydroxide

NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
s	Seconds
SD	Secure Digital
Taq	Thermostable DNA polymerase derivative of bacteria *Themus aquaticus*
TBE	Tris/Borate/EDTA
TM	Trademark
TSG	Tapir Specialist group
v	Volts

Chapter 1: Introduction

1.1 Biology of the Leopard (*Panthera pardus*)

The leopard (*Panthera pardus*) is the most extensively dispersed of the large felids, ranging from Africa to the Russian Far East (Mondol *et al*., 2009). The behavioural plasticity of leopards across various habitats is demonstrated by their broad geographic distribution (Jacobson *et al*., 2006). For example, leopards of the Kalahari Desert in Southern African survive without water for up to 10 days taking shade in the porcupine and aardvark burrows (Seidensticker & Lumpkin, 2004). Leopards in the far east of Russia will inhabit mountainous regions but are geographically limited by snow; avoiding areas with greater than 15 cm snow prolonged snow cover (Spitzen *et al*., 2012). Despite their natural elusiveness, increasing human population densities and habitat fragmentation are forcing leopard populations to become sympatric with human settlements, resulting in conflict, most often by livestock predation (Wilson & Mittermeier, 2009). For example, a study by Athreva *et al.* (2010) reported that in parts of India where fragmentation has caused leopards to become trapped in human-dominated landscapes, although human attacks are rare, livestock attacks are common. In mitigation efforts, the research found that translocation of individuals consequently induced attacks on humans, likely as a result of the stresses caused by the capture procedures. It was recommended that translocation was replaced with alternative control strategies such as better livestock protection and compensation to farmers following livestock mortality. In terms of human attacjks, although rare in most regions, one of the highest reported conflict zones for leopards attacking humans is in Gujarat, western India (Athreva *et al.,* 2007) whereby 847 incidents have been recorded between the years of 1992-2002 (Inskip & Zimmermann, 2009). Swanepoel *et al.* (2013) indicted that preferred habitat of the leopard tends to now be influenced by anthropogenic pressure, avoiding areas of high conflict where fragmentation doesn't restrict such evasion. The research suggested that in South Africa, mountainous areas are often inhabited by leopards where persecution is less intense. However, in areas absent of such conflict, such as in in privately-owned reserves, less-rugged terrain, and non-mountainous areas are often occupied.

Current morphological and genetic classifications describe nine subspecies of leopard (Stein *et al*., 2016). There is considerable variation in body size and weight, not just by sub-species but by region, with the Persian leopards (*P. pardus ciscaucasica*) considered the largest reporting males between 37-90 kg and females 28-60 kg across its range (Wilson & Mittermeier, 2009). In contrast, leopards of the Cape Province in South Africa are among the smallest exhibiting mean weights of 30.9 kg in

adult males and 21.2 kg in adult females, but with reports of males reaching up to 60 kg (Sunquist & Sunquist, 2002).

The leopards' broad distribution not only illustrates its variability in behaviour and size, but also in diet. Considered to have a true catholic diet (Stein *et al*., 2016), leopards' prey choice will consist primarily of small to medium sized mammals between 5 and 45 kg, although will occasionally take prey outside of this range. This includes mammals weighing less than 5 kg, aves, reptilians and even ungulates up to three times its own weight. In sub-Saharan Africa, the cats are known to prey on at least 92 species (Bailey, 1993). Leopards are primarily nocturnal, with infrequent hunting occurring during the day where dense cover prevails (Gavashelishvili & Lukarevskiy, 2008). It is thought that sharing a common habitat with other large carnivores can influence activity patterns of the leopard (Nowell & Jackson, 1996), promoting a temporal niche partitioning strategy in order to avoid interference competition (Dröge *et al*., 2017).

With the elusive nature of leopards making monitoring difficult, few studies have been conducted track the movements of leopards but home range is not thought to be restricted by the implementation of fence boundaries that are ubiquitous in South Africa (Hayward *et al*, 2008). A collation of literature by Wilson & Mittermeier (2009) suggests that home range and movement is predominantly associated with habitat (encompassing suitability and resource availability). In the Kalahari Desert, the greatest movement is reported with males travelling 14.3 km per day, and females with cubs travelling an average of 13.4 km per day. In habitats of higher productivity such as the South West Cape Province, no greater than 3 km was travelled by males in any one day. Furthermore, the home range of females in varying regions of low prey abundance could reach 487 km^2, yet females located in the riparian habitats of Kruger National Park averaged only 14.8 km^2. Adult males' home range is generally two to three times larger than that of females (Stein *et al*., 2011); driven by territorial and reproductive behaviour, to overlap that of one or two sexually mature females. Female leopards are considered philopatric, which often results in female offspring establishing home ranges adjacent to their mother's (Fattebert *et al*., 2015). Such a behavioural mechanism may hold potential implications towards the genetic health of a population, particularly if a breeding male holds a sympatric home range.

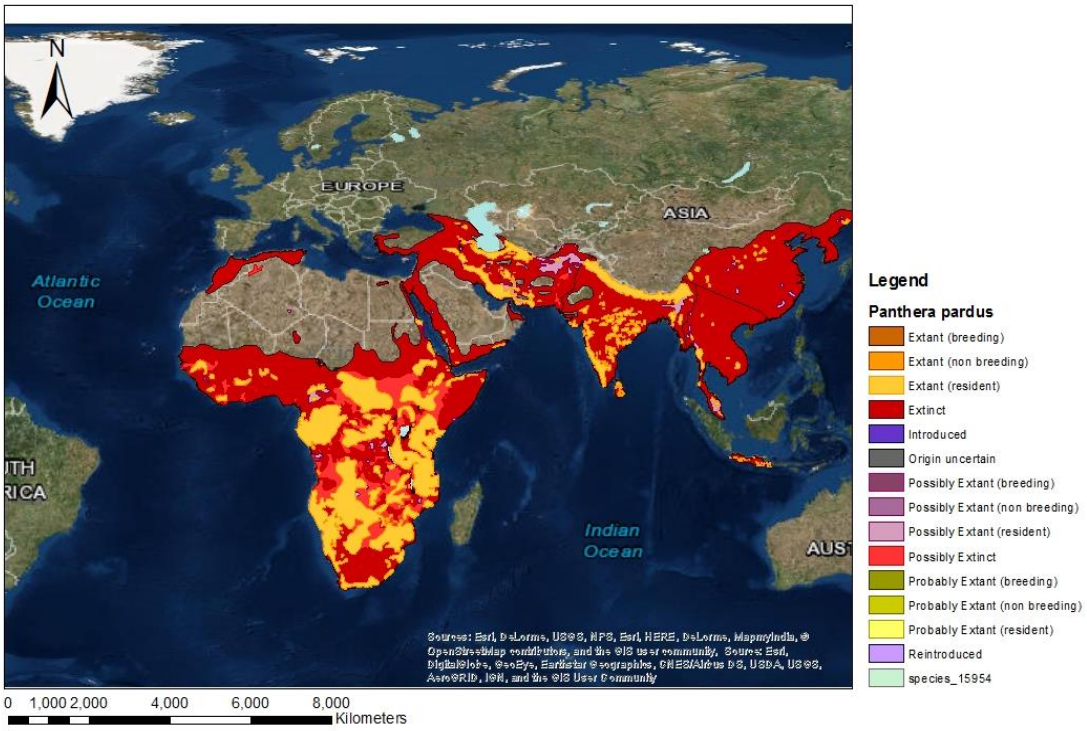

Figure 1.0. Global distribution and status of the leopard (*Panthera pardus*). Information and spatial mapping data taken available from The IUCN Red List of Threatened Species: *Panthera pardus* – leopard webpage.

1.2 Conservation and Genetics

The world's biological diversity is rapidly diminishing, largely as a consequence of direct and indirect human-induced pressure (Wilson & Mittermeier, 2009). The application of genetics into conservation biology materialised in the 1980s, when three publications recognised the potential for applying the principles of genetics in preserving biodiversity (Soulé & Wilcox, 1980; Frankel & Soulé, 1981; Schonewald-Cox *et al*., 1983). Since this time, genetic technologies have been applied with astounding precision; determining the effect of inbreeding, genetic drift and genetic diversity, and allowing interpretation of the health of individuals, species and populations at a molecular level. Conservation genetics is now a distinct scientific discipline encompassing ecology, evolution, molecular biology, population genetics and mathematical modelling to produce valuable quantitative data (Wan *et al*., 2004).

Genetics now plays a diverse and key role in conservation biology with recent advances such as the remarkable development of the Polymerase Chain Reaction (PCR) allowing amplification of specific

Deoxyribonucleic acid (DNA) target sequences. From this and the sequencing of the entire genomes of many species, the application genetics has revolutionised conservation biology (Mondol *et al*., 2009). Until recently, biological samples obtained intrusively have been heavily relied upon to provide sufficient genomic material. Destructive sampling whereby the animal is killed in order to obtain tissues has now been abandoned by many researchers (Taberlet *et al.,* 1999). Nevertheless, sampling for genetic analysis of deceased animals can include removals from organs, tissue and bone whilst common (non-destructive) invasive biological sample types taken from live animals again include tissue (often at a restricted size of around 20mg) and blood (Animal Care and Use Committee, 1998).

Animal tissue yields substantial quantities of DNA and as a sample type, is often deemed the highest in genetic quality (Roberts & Podesta, 2015). When addressing ecological questions, sequences taken from soft tissue samples can often provide remarkable information about the molecular dynamics of individuals and species that can be applied to an ecological understanding. For example, studies into mammalian migration, energy sources and even trophic position can be made from the stable isotopes (particular chemical elements) within soft tissue samples by acting as a tracer of information (Iverson *et al*., 2004; Thiemann *et al*., 2006). Soft tissue samples in particular are effective in this level of genetic application by having a high and variable isotope turnover rate between individuals, which can be related back to spatial and temporal differences (McFadden *et al*., 2006). Other sample types such as bone, hair and faeces contain a much lower isotope turnover rate (Tiezen *et al*., 1983), thus highlighting the importance of tissue as a sample choice in genetic analysis.

Tissue sampling can however encounter multiple restrictions and regulations in order to validate its use in research. This can include specific guidelines to limit the extent of harm that can be caused to the study subjects (Animal Care and Use Committee, 1998), and further procedures in transportations due to Convention on International Trade in Endangered Species of Wild Fauna and (CITES) and country-specific regulations (Berry & Aitken, 2007).

1.3 Non-Invasive Field Sampling of Wild Mammals

Traditional biological sampling for wildlife conservation research is often criticised for direct negative impacts on animals through excessive handling and sampling techniques that can not only harm the study subject(s), but potentially modify localised behaviour and wellbeing of the species (Taberlet *et al.*, 1999). The potential of such occurrence is greater when sampling methods require a large sample size, or procedures are replicated. As such, non-invasive sampling techniques have been progressively highlighted for their potential in addressing such concerns (Foran *et al.,* 1997). Relatively recent developments in molecular technology are also largely responsible for a drive in genetic sampling, thus adding to the importance of ethical considerations when obtaining biological samples (Panasci *et al*., 2011). However non-invasive field techniques are not as yet developed to a level of reliability to replace intrusive alternatives (Taberlet *et al.,* 1999; Roberts & Podesta, 2015). This absence of a recognised methodology highlights gaps in literature regarding not only the effectiveness of non-invasive sampling in genetic application, but in strategies of obtaining adequate samples. Obtaining biological samples of wild mammal species populations can be challenging particularly for those species which are elusive and/or widely dispersed (Busby *et al*., 2009). For such species, preliminary estimations of population abundance and distribution have conventionally been professed by non-direct means such as through tracks and signs, but these have often proved inaccurate upon more rigorous investigation (Belant *et al*., 2016). Camera-trapping strategies can ensure correct species categorisation but data is limited to camera placement (Du Preez *et al.,* 2014).

A non-invasive strategy utilised in order to avoid unnecessary handling of animals, is the collection of faecal samples. Considering the capture and handling guidelines set out by the Animal Care and Use Committee (1998), individual sample sizes can often be greater than invasive alternatives (such as live tissue sampling allowance). Faecal matter is considered easy to collect and can yield adequate, useable DNA (Wasser et al., 1997; Prugh *et al*., 2005; Bhagavatula & Singh, 2006). However, as Braczkowski *et al*. (2016) reported, the elusive nature of many wild felids can often produce a somewhat lacking sample size. A further limitation for advanced interpretation from faecal sampling is a lack of supporting visual data. It is most often unviable to set up recording equipment to gain phenotypic information when the potential excretion areas are extensive (Busby *et al*., 2009) and largely random. For this reason, faecal species-origin identification is traditionally conducted through morphological and other categorising features such as scent and content. Yet, according to Davison *et al.* (2002) misidentification of faecal samples has often been made where similar sized carnivores are sympatric. An example of this is in a pilot study by Bhagavatula & Singh (2006) that tested the

viability of faecal sample genotyping in estimation of Bengal tiger (*Panthera tigris tigris)* populations. When analysed in the laboratory, only 70% of all those collected samples that were truly of tiger origin, with others belonging to large carnivores of overlapping home range, particularly the leopard. Such findings not only reveal the extent of errors that can be made when using morphology alone in population estimates, but demonstrate the importance of genetics in supporting accurate research. With regards to transportation, permission to transport faecal samples varies per government and transport/shipping regulations. A CITES permit for faecal transportation is not required in South Africa for example, although if preservation occurs by ethanol, airline restrictions may be in place (TSG, n.d.).

Another non-invasive sampling option is that of hair samples, specifically targeting the follicle in which contains the connective living tissue, and therefore a source of DNA. Traditionally, hair snaring has long been used to obtain information about the presence of elusive mammals with species identification by macro and microscopic analysis (Braczkowski *et al*., 2016). Analysing hair samples in this way can often be ineffective particularly when closely related taxa are being examined, (Brunner & Coman, 1974; Friend, 1978; Valente & Woolley, 1982; Taylor, 1985). An investigation into the viability of this method was conducted by Lobert *et al*. (2001) and reported that 18 from a total of 37 mammalian species were subject to some extent of identification error. This factor when considered with the difficulties encountered in gaining hair samples in a way that can attain accurate DNA analysis may warrant investigation into the methodology behind in genetic sampling. Recently, hair capture in specific target of the follicle is promoted for its potential as a precise research tool where by animal capture is not required and the procedure can prove economically viable when material costs are low (Mestre *et al*., 2015). Success rates of hair snaring have shown to be inconsistent, most likely due to variable pilot methodologies, effort, and species-specific adjustments (Zalewski, 1994).

Previous methods of obtaining hair samples from wild mammals have seen the use of various techniques such as scent and bait lures (Patkó *et al*., 2015, 2016) to barbed wire (Foran *et al*., 1997), and barbed rubbing pad devices, with varying degrees of success and no current standardised technique. Patkó *et al*. (2016) indicated that more than one bait or scent attractant is often required for effective carnivore attraction, and findings of Braczkowski *et al.* (2016) suggested that the use of scent lures for leopards is inconsistent in results, and therefore not warranted for future research. Data is limited in the consistency of mechanisms used for wild felids, yet it seems a barbed device has been the most heavily relied upon method in obtaining hair and follicle samples (Clevenger & Sawaya, 2010; U.S Fish and Wildlife Service, 2017). These hair-trapping techniques may be regarded as ethical in the absence of direct human contact, but mechanisms that consist of barbed devices

can cause some damage, or risk of harm to the target animal. With increased genetic sampling occurring as conservation genetics becomes established as a reliable tool, ethical considerations should appropriately correspond with such advances.

Other methods trialed for hair-capture that further minimise the risk of injury to animal include adhesive mechanisms such as glue boards and adhesive tape. Glue boards have been successfully used to obtain hair samples, but have encountered sample interference where the glue itself inhibits the usability of the follicle (Mowat & Paetkau, 2000; Sloane *et al*., 2000, Frantz *et al*., 2003, Long *et al*., 2008). A similar approach, but alternative technique is that of adhesive tape. Trialing of this method is limited, yet has shown huge success in understanding population dynamics of the Southern hairy-nosed wombat (*Lasiorhinus latifrons*) (Walker *et al*., 2006) and the Northern hairy-nosed wombat (*Lasiorhinus krefftii*) (Sloane *et al.,* 2000), with sample collection occurring by strategic tape placement at burrow access routes. Such sampling techniques can be considered more sensitive to the ethics of study subjects, but with any adhesive constituent, steps should be taken to avoid removal or interference of sensory hairs, specifically facial hairs and whiskers (Animal Care and Use Committee, 1998). With the ethics, precision and effectiveness supported, this study may warrant further investigation the strategy in aim of establishing a standardised technique. Furthermore, hair as a sample choice is considered easy to transport in comparison to other biological samples of which require appropriate import-export permits (TSG, n.d).

1.4 Non-Invasive Genetics

Despite the increasing use of DNA in ecological understanding, and highlighted importance on genetics in conservation biology as described by Allendorf & Luikart (2007), the use of non-invasive genetic data in research is still relatively novel. Developing usable genetic information requires scrutiny in processes of handling, storage and laboratory applications according to particular sample types. DNA obtained from non-invasive samples can be particularly subject to genotyping errors (Gerloff *et al*., 1995; Goossens*, et al*., 1998; Taberlet *et al*., 1999 and Piggott & Taylor, 2003) often through sample deterioration, inhibitors and lack of optimal sample processing techniques (such as storage, DNA extraction and amplification). These errors are often the result of allelic dropout, where an allele of a heterozygous individual is not amplified during PCR, and false alleles, of which are generated during PCR as a result of a slippage (Broquet & Petit, 2004). These two sources of error are not easily monitored and so can present important confounding factors that pose risks of inaccurate study outcomes (Gerloff *et al*., 1995; Taberlet *et al.,* 1996). Regardless of sample type,

steps can be taken to limit the extent of DNA degradation before extraction. This can include ensuring all field and laboratory processing techniques such as handling, storage type and time in storage are kept within the optimal conditions according to sample type collected (Taberlet *et al*., 1999).

The importance of genetics in supporting ecological research has been described in studies such as Bhagavatula & Singh's (2006) increased accuracy in Bengal tiger population estimations when genetics are applied, demonstrating the effectiveness of faecal sampling. Further support of this sample choice is seen in a variety of mammalian test subject species such as DNA application tests in members of the *Ursidae* family (Wasser *et al*., 1997), population abundance in the European badger (*Meles meles*) (Wilson *et al*., 2003) and population dynamics of the coyote (*Canis latrans*) (Prugh *et al*., 2005). The research of Wasser *et al*. (1997) reported no significant difference in individuals' genotyping between faeces, tissue and blood, thus strongly supporting the non-invasive sample choice as a suitable strategy. A driver behind this research was a limitation in understanding of appropriate DNA techniques to support of field-based studies, thus demonstrating that optimal faecal collection and preservation procedures for DNA genotyping are not yet firmly established. Inhibitors in faecal matter are described as important restrictors with regards to genetic applications such as DNA amplification, yet current knowledge is lacking (Penasci *et al.*, 2011). To optimize laboratory performance, only the outer sample layers of a faecal sample that have made contact with the intestinal epithelial cells of the subject animal should be targeted (Piggott & Taylor, 2003; Bhagavatula & Singh, 2006). Furthermore, it was found by Wehausen *et al.* (2004) that the DNA extracted from this part of the sample has less PCR inhibitory substances and risk of contamination from detectable prey remains of carnivores. Nevertheless, if the outer layer has encountered fungi growth, interference from other organisms, and interruptive environmental conditions such as rainfall, DNA extraction is likely to be considerably hindered. On the other hand, a consistently dry environment such as the arid seasonal conditions of Southern Africa can naturally preserve the sample and DNA content (Taberlet *et al*., 1999).

For the best chance of successful DNA extraction, faecal samples must be as fresh as possible upon collection with suitable storage processing conducted rapidly to minimize effects of further interference (U.S. Fish & Wildlife Service, 2017). Research dependent upon faecal collection, where samples have been exposed to variable environmental conditions over a long period of time can suffer a dramatic reduction of quantifiable samples in lab processing (Petit & Valiere, 2006). This is a factor that must be taken into consideration in any sampling design where reliance is placed upon this strategy. As a guide, it has been recommended by Penasci *et al*. (2011) in a study of faecal

genotyping that DNA can be most easily and economically obtained when faecal samples are less than five days old upon collection. Those that have been exposed to the environment for longer than this can still produce reliable results in laboratory analysis, yet may require more precise preservation, extraction and amplification techniques at a higher cost.

During storage of any sample type it is essential that the opportunity for nucleases to degrade the DNA within the sample is reduced as much as possible. This is achieved by ensuring the molecular environment surrounding the sample is maintained in such a way that it is inoperative to enzymatic activity (Piggott & Taylor, 2003). Storage methods that restrict such an event have been used to variable success rates with some studies deeming techniques optimal in some instances, whilst others have achieved poor DNA performance (Taberlet *et al.,* 1999; Waits & Paetkau, 2005). The guidelines of Penasco *et al*. (2011) proposed that the most reliable preservation technique for carnivorous mammals is DET (consisting of Dimethyl sulfoxide; EDTA; Tris; sodium chloride) buffer, 95% ethanol (EtOH) preservation, yet there is a striking lack of consistency between preservation applications when reviewing mammalian faecal storage in general (U.S. Fish & Wildlife Service, 2017). For instance, silica desiccant proved optimal in Black bear (*Ursus americanus*) genotyping (Wasser *et al*., 1997), which was also the case for analysis of Jaguar (*Panthera onca*) and Puma (*Puma concolor*) (Farrell *et al*., 2000), but least effective in Brown bear (*Ursus arctos*) genotyping (Murphy *et al*., 2002). Storage in ethanol produced poor results for the *Ursidae* studied by Wasser *et al*. (1997), yet reliable for Piggot & Taylor (2003) in a study specifically analysing remote collection methods in understanding the genomics of elusive species. It appears that methods of desiccation by silica gel beads and by 90% ethanol worked to a similar and effective level in Bhagavatula & Singh's (2006) research of Bengal tigers, with suitability extending to those other sympatric carnivores species incorrectly sampled in the field. In some instances, such as the study of Prugh *et al*. (2005) into coyotes, preservation by freezing occurred instantly upon defecation due to winter temperatures, with success in laboratory analysis. Murphy (2002) recommended storing samples in 90% ethanol when feasible, or replacing with a desiccation technique in remote field conditions where long-term storage or aged samples are likely. It has been indicated in a review of these different methods of preservation for genetic management by the U.S. Fish & Wildlife Service (2017), that it appears optimal conditions of storage can be largely dependent of species, feeding guild (variations in lipid content), environmental climate, and local conditions. Further implications include time in environment before collection (Penasci *et al*., 2011) and period of storage (Taberlet *et al.,* 1999). Further to these considerations, Piggott & Taylor (2003) also reported an association between storage technique and extraction methods in the laboratory by which particular extractions performed better according to specific storage procedures. Again, these confounding factors can

warrant the necessity in investigation of non-invasive genetics with particular regards to various faecal storage and extraction techniques according to subject species and conditions. Despite inconsistencies it appears from the mentioned literature that the most effective faecal preservations appropriate to carnivores of arid conditions are by ethanol and desiccation techniques.

Less is known about preserving hair samples than faecal (U.S. Fish & Wildlife Service, 2017) yet hair samples have performed well in terms of both quantity and quality of DNA extracted (TSG, n.d.). For DNA analysis, literature generally supports the preservation of hair samples in a desiccated state (Taberlet *et al.*, 1999; U.S Fish & Wildlife Service, 2017). Roon *et al.,* (2003) compared the preservation of hair from captive mammals for mitochondrial DNA analysis, testing samples stored by silica desiccant against those maintained at -20°C. The study identified no significant difference in DNA extraction success rates between the techniques. An important consideration when choosing to freeze samples, especially with regards to necessary transportation by which desired storage conditions may not be attainable; is the potential freeze-thaw effect cleaving the DNA (TSG., n.d.). In field situations where equipment may be limited and the freezing of samples is not an option, studies have generally supported hair preservation in silica desiccant or in paper envelopes (Mowat & Paetkau, 2000; Sloane *et al*., 2000; Long *et al*., 2008). The method of desiccation should reflect the requirements that the field environment presents. For instance, a humid environment may not maintain those hair follicles by air-drying alone, and can require artificial substances such as silica desiccation beads that come equipped with chemical indictors of moisture saturation (U.S. Fish & Wildlife Services, 2017). In arid conditions, storage in paper envelopes has been conducted at no extra detriment to the sample than with silica beads (Taberlet *et al.*, 1999; TSG., n.d.).

Previous research that has relied upon hair samples does not report the inhibitory problems that persist with faecal samples in genetic analysis, yet the errors in genotyping are problematic in non-invasive sampling as a whole (Busby *et al*., 2009), so this remains a complication. A study by Walker *et al.,* (2006) into the ranging behaviour of a population of Southern hairy-nosed wombats encountered little loss of sample size in laboratory analysis. This study extracted DNA promptly after collection (within 24 hours) in the field location, thus suggesting storage could be a major implicating factor of those studies that have suffered sample size reduction (García-Alaníz *et al.*, 2010; Stenglein *et al*., 2011).

Broquet *et al.,* (2006) concluded that the choice of genetic marker, i.e. the identifiable region of genetic material, is a factor that largely optimises the success rates of non-invasive research. The research indicated that short markers at around 100-150 base pair (bp) length are best suited to the smaller size of non-invasive samples that are at greater risk of being degraded or fragmented.

Further to this, the suitability of the primers (forward and reverse short nucleotide sequences from which DNA replication can begin and end) that are chosen are an important consideration with regards to the genetic marker, and outcome aim. The importance of primer specificity was described by Wilcox *et al.* (2013) in a study that investigated the use of primers in analysis of endangered species using environmental DNA (eDNA). The research found that bias in species detection was most influenced by nucleotide pair mismatches in the primers, and such results posed a substantial risk of false positives, thus leading to overestimation of endangered species populations, or false negatives preventing detection of potentially threatening invasive species. Reliability of primers can be dependent upon sample type and target species, such as the faecal analysis of carnivore diet by Chaves *et al.* (2011) which found that carnivore-specific adenosine triphosphate 6 (*ATP6*) primers were most effective in eliminating prey contamination when compared against *cytochrome c oxidase subunit I* (*COI*) primers. However, alterations to the nucleotide design of the latter choice were able to improve the quality and produce the same specificity in results. Primer availability is increasing in line with its progressively common use in wildlife genetics; whereby primer selection largely relates to the specificity required for the particular study (Li *et al*., 2008). For example, reliable primers can be universal and sensitive to a wide range of sympatric species (Branicki *et al.,* 2003), carnivore-specific (*Carnivora*, 2011; Chaves *et al*., 2011) or designed by the researcher to distinguish a particular genus or species (Nagata *et al.,* 2005; Bhagavatula & Singh, 2006; Janečka *et al*., 2008). In other words, the choice of both genetic marker and primer design must take into account the individual or species being analysed, and to which extent of genetic examination.

Wang & Smith (2014) conducted the first test of the precision of non-invasive sampling in more scrutinous downstream genetic applications, rather than just looking at species identification alone for example. The research found that accurate information on expected heterozygosity, fixation index (FST), population structure and allelic diversity could be obtained with little bias, but only with a sample size of ten or above. With the loss of sample size that should be expected, as with the Bhagavatula & Singh (2006) non-invasive sampling research, this is a factor that should be accounted field data collection, i.e. sampling procedures should not be discontinued once a desired number of collections are made, as this number is likely to be reduced upon laboratory analysis.

The potential for non-invasive genetics is apparent but a number of factors must be taken into consideration for any research reliant on indirect sampling. From the sample choice, sample size, collection, and preservation through to appropriate laboratory applications, these components should all be pre-assessed to determine the degree of uncertainty that can influence the hypothesised ecological question.

1.5 Hypothesis and Project Aims

The hypothesis of this study is that adequate genetic information can be gained from non-invasive sampling if the appropriate field techniques and optimal processing methods are applied.

Based upon the need to further investigate the viability of non-invasive sampling in genetic analysis, and the need for a replicable field sampling technique; this project sets out to achieve the following aims:

1. Compare biological sample types for genetic analysis through quality and quantity of DNA.
2. Propose and implement a new technique for non-invasive sampling of African leopards (*Panthera pardus pardus*).
3. Explore the efficiency of non-invasive samples to genetic applications.

Outcomes of research may assist in investigating the viability of different biological sample types, present an effective method for non-invasive genetic sampling of elusive species, and enhance data available in non-invasive genetics. Further research when genetic information on a species is obtained can be applied to conservation management strategies if deemed necessary.

Chapter 2. Methodology

Methodology consisted of both field and laboratory elements with data collection occurring in the field within South Africa during a six-week period from May 28th 2016 during the dry season, and further genetic developments of samples conducted in the University of South Wales lab.

2.1 Field Study Site

The Selati Game Reserve is a 29, 411 ha private "closed system" reserve (Selati Game Reserve, 2017) situated in the Limpopo Province in the north east of South Africa (fig. 2.1) Founded in 1993, the reserve management aims uphold the well-being of the ecosystem through conserving the dynamic interactions between local climate, geography and the populations of both flora and fauna species. With effective management decisions relying upon understanding of this interplay, this study assists in providing a scientific approach to a relatively understudied area (Joubert, 2016) by providing preliminary investigations into the felids of the reserve.

The reserve takes its name from the Great Selati River which meanders for approximately 22 km east to west through the northern part of reserve, and is situated in the South African Lowveld between longitudes 30° 38' 42" E and 30° 54' 26" E and latitudes 23° 54' 25" S and 24° 05' 09" S. Gravel roads form the northern and southern boundaries of the reserve, whilst farmland borders the eastern edge. The western borders are produced by the Gravelotte Emerald mine and Gravelotte municipality (Joubert and Joubert, 2015).

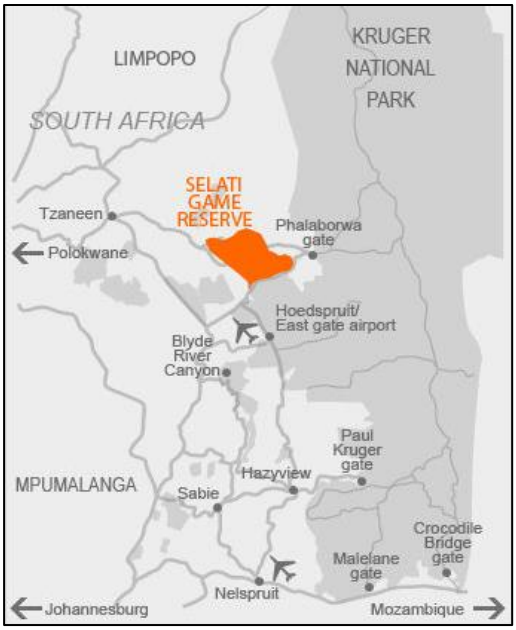

Figure 2.1. Location of Selati Game Reserve in South Africa.

Selati Game Reserve falls within the hot, semi-arid steppe climate zone. The climate is typically dry with a mean annual temperature exceeding 18°C and a mean annual rainfall of 530 mm (Peel *et al.,* 2007). Consisting of both reserve-owned and privately owned land with 22 farming units, the reserve comprises undulating topography of agricultural land and natural features producing a mosaic in both landscape and biodiversity. The reserve has three major granite and pegmatite formations; the Willie, Lekkersmaak and Mashishimale gneisses, with the Murchison Greenstone belt in the north-west containing some of the oldest rock formations on earth (Joubert and Joubert, 2015). The well-established geological features support six major plant communities. Mixed *Combretum* veld dominates the high areas; *Terminalia* trees, the poorer soil areas and *Colophospermum mopane* woodland dominating the clay soils associated with drainage lines and low lying areas. The reserve is well known for its cycad community with particular focus upon cycad *Encephalartos dyerianus*, the only naturally occurring colony of this species on earth (Limpopo DFED, 2004).

The reserve holds over 280 bird species and 50 species of medium to large mammals, some of which have been long-established and others that have been brought in to boost populations and improve the gene pool of existing species. The biodiversity has been largely enhanced through the reintroduction of African elephant (*Loxodonta*) and African lion (*Panthera leo*). Other mammals of the site include the African leopard (*Panthera pardus pardus*); African cheetah (*Acinonyx jubatus*); Wild dog (*Lycaon pictus*); Spotted hyena (*Crocuta crocuta*); Honey badger (*Mellivora capensis*); Sable antelope (*Hippotragus niger*) and Common eland (*Taurotragus oryx*). Due to unfavourable conditions associated with the dry season, the Selati Game Reserve management conducted the removal of

several mammals from March 2016 including 373 impala (*Aepyceros melampus*); 149 Blue wildebeest (*Connochaetes taurinus*) and 24 Plains zebra (*Equus quagga*) (Joubert & Joubert, 2015).

2.2 Fields Methods: Lion Sample Collection

Lion samples were used to provide the genetic comparison of DNA quality and quantity obtained from different sample types.

2.2.1 Faecal matter

One male and three females (recorded as 1:3) lions were tracked to sampling location (fig. 2.4) by radio telemetry device (Comunications Specialists R-1000 Telemetry and Handheld Receiver) and fresh faeces from 1:1 animals were collected after the individuals moved away from the site. Prior to collection, safe distance was determined through use of the telemetry device.

To ensure optimal chance of DNA extraction upon laboratory analysis, three storage methods were conducted per faecal sample. Sample storage processing was conducted immediately on site by use of a scalpel and blunt-tipped forceps to remove approximately 20 mg (estimated on site) of the outer coating of the sample into each of the three sterile 1.5 ml Biopur® Eppendorf tubes. Two tubes were filled to 1ml with 70% ethanol (Alpha® Surgical Spirit Chirurgiese Alcohol), and the remaining filled to 1ml with 40 % vodka to ensure full immersion of the faecal matter. The lids were sealed securely and Parafilm M® was wrapped around the lid of each.

Upon return to base camp, one ethanol-filled Eppendorf was stored at -20°C and the vodka-filled tube stored at room temperature. The other ethanol-filled Eppendorf was left at room temperature for 48 hours (minimum), before being drained and desiccated by insertion of 1 cm x 1 cm cut Rombats® filter paper and around 10 Odourvek® silica gel beads. Parafilm was re-applied to the desiccated sample and tubes were placed within zip-lock bags to be stored in this way until laboratory analysis.

2.2.2 Tissue

Tracking of 1:2 lions was conducted by radio telemetry to provide sampling location (fig 2.4). The method of biopsy drop-out darting as approved by the Reserve Management was conducted by a licensed firearms holder on 1:1 lions. The dart units were collected by the firearms holder once the animals had moved away from the site. The complete dart unit was placed into a sterile bag on site, sealed and both samples taken to the researchers' base camp site for processing.

At base camp, aseptic techniques were used to take the dart apart and remove the tissue from the two locking hooks (fig. 2.2). The whole sample was placed into a 1.5ml sterile Eppendorf, filled until completely immersed in 70% ethanol, sealed securely and wrapped in parafilm for storage until laboratory analysis.

Figure 2.2. Drop-out biopsy dart assembly. Complete unit (A), Locking hooks on needle that secure sample upon tissue penetration (B).

2.2.3 Hair

1:0 Lion hair sample was obtained by the Field Research Technician of Selati Game Reserve later than the initial six-week data collection period during an operation in which 1:0 lion needed to be anaesthetised. The sample was stored in an airtight Falcon tube (Fisher Scientific) with desiccating silica gel beads until laboratory analysis.

2.3 Field methods: Non-Invasive Sampling of Leopards

2.3.1 Baited tree mechanism

Bait trapping locations were set out as per a stratified random sampling system, described by reserve management across Selati Game Reserve (fig. 2.4). A game viewing off-road safari vehicle was used to transport a minimum of two people to each bait site location. Within each area, bait tree choice was made by identification of a suitable mopane tree with three or greater primary branching forks. Consideration was also taken for an adjacent tree to allow appropriate camera trap placement, with direction of capture avoiding sunrise and sunset camera lens glare; and a distance of 5m (or as near to as possible) from the bait tree, in accordance with camera model suitability.

A mopane tree was cut down and all small branches removed to create the leopard entry pole. This pole was placed from the ground to the lowest fork to as close to a 45° angle as possible (fig. 2.3). A quarter of a larger bait (prey species zebra/ male impala), or half of a smaller bait (prey species female impala was prepared by using a double layer of flexible metal wire through the meat and around the vertebrate spine of the bait. By climbing the tree, the bait was then secured around a higher branch (wider than 10 cm diameter) for bait placement in an area central to the tree forking system. This was for the intention of producing a natural passage that the leopard needs to enter for the potential of hair removal by bark-rubbing. Due to natural variation of tree morphology, bait was adjusted to suit each bait site according to the natural passage by which the leopard was intended to move through.

Adhesive Stikka® duct tape was placed across the main forking system by which the leopard would move through in order to retrieve the bait. Additional duct tape was lined against the longitudinal surfaces of the branches as a means of providing a secondary sampling surface the leopard would rub against when moving through to obtain the bait (fig. 2.3). The duct tape was secured either by wrapping further tape length around the supporting branch or by use of flexible wire for wide or difficult-to-access branch networks.

The intention when placing duct tape was to consider the area that the leopard would lean into or move against with suitable force to allow adhesion of hairs and adequate pull in order to obtain the full follicle in that sample. The adhesive surface should hold the outer section of hair, thus leaving the DNA-containing follicle exposed and intact.

After the bait trap mechanism was in place, a pelt of the prey item was used to create a scent trail. The pelt was kept saturated in a bucket until required, then attached to the back of the vehicle by wire to be dragged away from the site for one kilometre along the closest road.

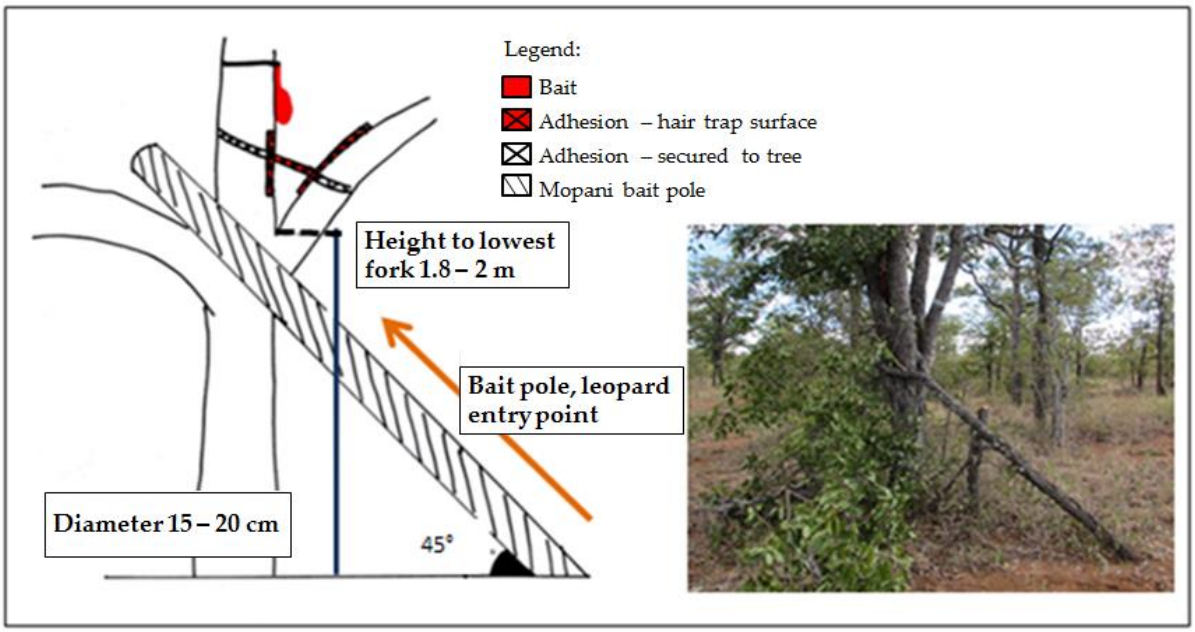

Figure 2.3. Diagram of basic bait tree system targeted at obtaining hair samples from the African leopard (*Panthera pardus pardus*) (left). Mopani bait tree species with three or greater primary branching network chosen. Pole placement of 45° angle from ground to lowest branch fork. Bait was secured by flexible wire. Duct tape secured by wire/excess duct tape depending on diameter of attachment branch. Comparison photograph (right) shows mechanism in the field. Natural obstructions were applied to ensure leopards' path follows desired entry point.

The baiting method described was in place for two weeks per bait site, a time period of which was less than optimal considering some leopards are readily attracted to bait in later stages of decay (Bailey, 1993). The baiting period is reflected by replacement of an original system made up of two primary branches (to hold the pole in place) and razor wire to obtain hair. This method proved ineffective in obtaining hairs with follicles and so was terminated after a period of three weeks. Data from the original method were disregarded for this study after laboratory applications were trialled and found to be ineffective at extracting DNA.

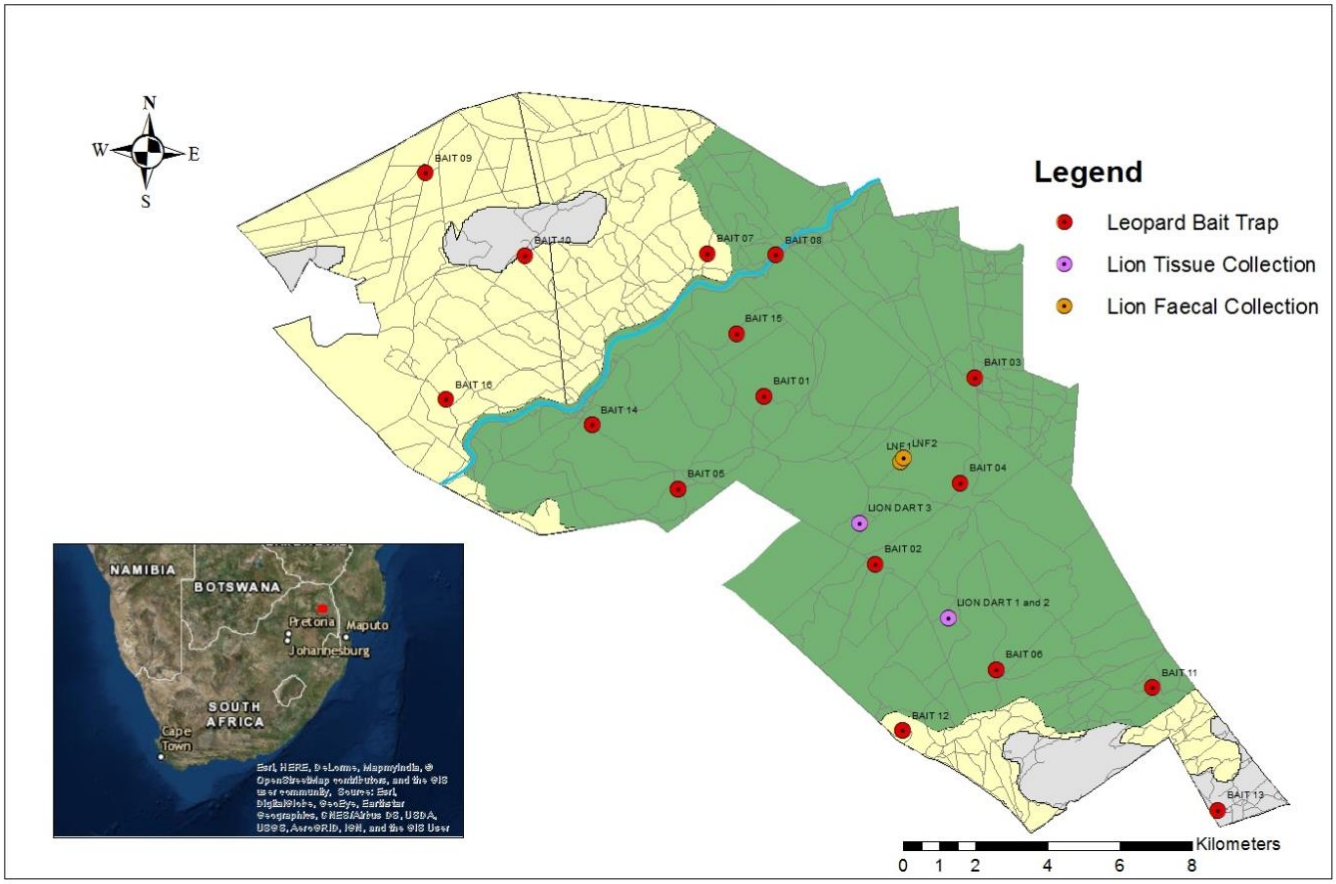

Figure 2.4. African lion (*Panthera leo*) faecal and tissue collection sample points and African leopard (*Panthera pardus pardus*) sampling design across the Selati Game Reserve, South Africa

2.3.2 Camera trap data collection

For visual evidence and phenotypic identification of leopards, Bushnell® Trophy Cam Aggressor HD camera traps containing 8GB Secure Digital (SD) memory cards were placed in the appropriate position to capture the right flank of the leopard when on the bait entry pole. The following adjustments/verifications were made to the camera trap settings:

- 8M pixels
- Full screen view
- Photo sequence = 3
- LED control = High
- Interval = 5 seconds
- Sensor level = Normal
- Night vision shutter speed = normal
- Mode = 24 hour recording

The following information was recorded at each bait trap site:

- Date
- GPS coordinates using a Garmin e-Trex® 30x Global Positioning System portable device.
- Grid reference of Selati Game Reserve.
- Bait tree number and name (location name used).
- Freshness of meat on placement.
- Notes: Including bait used, any alterations to suit bait tree choice, conditions on the day

Leopards were identified and distinguished by rosette patterns on analysis of camera trap data. The most consistent area of phenotypic comparability was the hind region of the leopards' right flank (fig. 2.4). However, one leopard was identified by a radio-collar (non-functioning) as the distinguishing feature, and individuals that cannot be identified as new or existing individuals are specified as such in the results section. All complete camera trap photos used for identification are available in appendix VIII.

Bait traps were checked every two-three days. Camera trap data was first checked at the bait site by removing the SD card from the camera trap and checking the photos on a portable laptop computer.

Figure 2.5. Example of the area used for phenotypic identification. Individual L4. Location: Bait trap 3 Steve's Cutline. Identifiable rosette markings of right flank on 1:0 leopard circled.

2.3.3 Sample collection

If a leopard was identified on the visual data, the bait tree was checked for hair samples. Hairs on duct tape were removed with bait strap system still in place. Sterile blunt-tipped forceps were used to pluck individual hairs of which had exposed follicles from the duct tape, and immediately placed into a 10 x 5 cm unwaxed envelope. The hairs that appeared to be of the highest quality (i.e. follicle exposed and untouched whilst outer section of hair secured to tape) were removed and sealed in the envelope first. The duct tape was then removed carefully, with scissors if necessary and placed into clear zip lock plastic bag. Final checks for hairs were made in and around the bait site branches and ground area, with such samples stored separately in a new envelope and labelled according to area of collection. As a secondary storage method, when sample collection provided greater than 20 hairs, upon return to base camp up to 10 hairs were placed into a sterile 1.5 ml Eppendorf and filled to 0.5 ml 70% ethanol, with those excess remaining on the duct tape and in the zip lock bag, the purpose of which was to enable further extraction of hairs if required in a sterile environment.

When visual data indicated bait interference of other mammal species, or more than one leopard, the areas in which each individual leopard made contact was identified (with the assistance of visual data) and sampled. Hair sample collection in cases where distinguishing areas of contact according to species was difficult, was conducted by separating hairs according to similar features.

Envelopes were labelled with the following:

- Date
- Researcher name
- Bait trap number and location
- Species interference: identified from camera trap data
- Number of sample collections from that site
- Number of that particular sample (e.g. 1 of 1; 1 of 4)

When no interference by leopard was determined by visual data, the duct tape was removed and replaced if necessary (i.e. environmental conditions or other species inhibited the adhesion of the tape). If bait was absent or consumed (to a state where mainly bone remained), the bait system and camera trap was removed. All camera trap data were transferred to a laptop on site regardless of leopard presence or absence, to then be formatted in the camera trap setup options, and replaced.

2.4 Laboratory Methods

2.4.1 DNA extraction

Three extraction methods were used for the leopard and lion biological samples, and the optimal method for each sample type was indicated in the results (chapter four).

2.4.1.1 Extraction one

Tissue extraction occurred by use of QIAamp® DNA Investigator Kit (QIAGEN®). DNA was purified and extracted as per protocol: Wizard® SV Genomic DNA Purification System: Quick Protocol (Promega, 2012) with the following notes and adjustments:

Step 13. This step skipped (repeat of step 11).

Storage: Storage remains at -20°C until required.

For the complete protocol see Appendix I.

2.4.1.2 Extraction two

Faecal extraction occurred by use of QIAamp® DNA Stool Mini Kit (QIAGEN®). DNA was purified and extracted as per protocol: Isolation of DNA from Stool for Human DNA Analysis (QIAGEN, 2012) with the following notes and adjustments:

Step 2. Samples were vortexed in buffer ASL for 5 minutes.

Step 5. 0.5 InhibitEX® (QIAGEN®) tablet added to each sample.

Storage: Storage remains at -20°C until required.

For the complete protocol see Appendix II

2.4.1.3 Extraction three:

Hair extraction occurred by use of QIAamp® DNA Investigator Kit (QIAGEN®). DNA was purified and extracted as per protocol: Isolation of Total DNA from Nail Clippings and Hair (QIAGEN, 2012) with the following notes and adjustments:

Step 1. 1b used for hair roots. Hair was cut by aseptic techniques using pointed forceps to grip the desired section of hair (not in contact with follicle), whilst cutting to 0.5-1 cm length.

Step 17. Final incubation conducted for 5 minutes at room temperature to maximise DNA yield

For the complete protocol see Appendix III

2.4.1.4 Extraction four:

Hair extraction occurred by Cetyltrimethyl ammonium bromide (CTAB) method was used to try to gain higher quality and quantity DNA extraction. DNA was purified and extracted as per a CTAB protocol (Doyle and Doyle, 1987). For the complete protocol see Appendix IV with stock solution constituents listed in Appendix V.

2.4.2 DNA quantification

Quantity and purity of the extracted DNA was checked on the NanoDrop™ 2000 spectrophotometer (Thermos Scientific™). The Nanodrop™ 2000 software programme was used to visualise and record results. The ng/µl unit of concentration was recorded to quantity DNA of each sample and ratios (260/280 and 260/230) were recorded to determine the purity of DNA and identify evidence of contaminants.

For each sample, the pedestal of the spectrophotometer was wiped with a lens cleansing wipe. The final buffer used in each DNA elution was used to blank the Nanodrop machine by loading 1µl buffer directly onto the pedestal, taking care not to touch the instrument with the pipette tip. After blanking the read, the pedestal was again wiped and 1µl sample loaded.

Guidelines for interpreting Nanodrop readings were as follows:

Conc. = Concentration of DNA in sample (ng/µl). Sufficient quantity of DNA for amplification should usually be 5 ng/µl or greater.

260/280 = Indicates purity of DNA. A pure DNA reading indicated by a value within 1.8 – 2. A lower value indicates possible contaminants and changes of acidity in nucleic mix.

260/230 = A secondary indicator of purity. A pure DNA reading indicated by a value between 2 – 2.2. A lower value indicates possible contamination from Ethylenediaminetetraacetic acid (EDTA), carbohydrates and phenol contaminants.

Ratios of greater than 2.0 indicate the potential presence of ribonucleic acid (RNA) within the extracted sample (Thermo Scientific, 2008). Full details are provided in Appendix VI.

The measurements produced by the Nanodrop programme were recorded for each DNA extraction sample into a researcher laboratory book.

2.4.3 PCR process

Amplifications were performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems™). All primer sequences were provided by Sigma-Aldrich® and all other PCR constituents were provided from a Taq PCR Kit (New England Biolabs®**)** unless otherwise stated.

To optimise chances of mitochondrial DNA (MtDNA) target region amplification, PCR programmes were made using carnivore-specific primers *COI* based upon previous felid research by *Carnivora* (2011), and *ATP6* (Chaves *et al.,* 2011; Michalski *et al.*, 2011). In addition, a universal primer cytochrome b (*cytb*) was used following research by Forgham *et al*. (2015) and Branicki *et al*. (2016). A total of seven PCR trials were applied to DNA samples, and those which produced successful amplification are described here in the methodology. All other trials are available in Appendix VII. A negative control was included in every PCR trial which was processed in the same way as the extracted samples, but absent the template DNA. For PCR trials that required more than one electrophoresis gel, additional negative controls, containing the same 6x dye and nuclease-free water were prepared. This was to ensure all gels had a negative control and also identify primer position in relation to any DNA bands.

2.4.3.1 PCR one

ATP6 primers (126 bp length):

Forward primer sequence: 5'-AACGAAAATCTATTCGCCTCT-3'

Reverse primer sequence: 5'-CCAGTATTTGTTTTGATGTTAGTTG-3'

PCR master mix per sample:

Taq buffer	2.5 µl
Deoxynucleotides (DNTPs)	0.5 µl
Taq polymerase	1 µl
Forward primer (diluted 1:10 original stock solution: Nuclease-free water)	0.5 µl
Reverse primer (diluted 1:10 original stock solution: Nuclease-free water)	0.5 µl

Between 1 – 5 µl of extracted DNA template is also added based upon ng/µl Nanodrop reading per sample. Up to but no greater than 250 ng/µl is added to each sample. Dilutions were adjusted in further PCR trials (1:10 and 1:5).

Each sample of master mix, DNA template is pipetted into sterile PCR tubes and each filled to a total volume of 25 µl. A negative control with no DNA template was added with each PCR operation.

PCR programming (Chaves *et al*., 2011):

Initial denaturation: 94°C/3’

Denaturation: 94°C/45”

Annealing: Touchdown cycle: 60°C/45” (touchdown -1°C/10 cycles)

Elongation: 72°C/ 1’30”

Further 30 cycles: 94°C/45”, 50°C/45”, and 72°C/1’30”

Final elongation: 72°C/3’

Storage: 4°C on infinite setting (until removal).

2.4.3.2 PCR two

COI primers (187 bp length):

Forward primer sequence: 5'-CCCCTATTCGTATGATCAGTATTAATTAC-3'

Reverse primer sequence: 5'-TAAACCTCAGGATGTCCGAAGAATCA-3'

PCR master mix and addition of DNA template as per the *ATP6* primer instructions.

PCR programming (*Carnivora*, 2011):

Initial denaturation: 96°C/1'

Denaturation: 94°C/20"

Touchdown 40 cycles: 94°C/30", 50°C/5", 72°C 1'30" (annealing temperature reduced by 2°C until final annealing temperatures of 40°C after 10; 5; 5; 5; 5; 10 cycles)

Final elongation: 72°C/3'

Storage: 4°C on infinite setting (until removal).

2.4.3.3 PCR three

Cytb primers (358 bp length):

Forward primer: 5'-CCATCCAACATCTCCGCATGATGAAA-3'

Reverse primer: 5'-CCCCTCAGAATGATATTTGGCCTCA-3'

PCR master mix and addition of DNA template produced as per the *ATP6* primer instructions.

PCR programming (Branicki *et al*., 2003):

Initial denaturation: 94°C/2'

Denaturation: 94°C/20"

Annealing: 51°C/30"

Elongation: 72°C/40" (36 cycles of denaturation, annealing, elongation)

Final elongation: 72°C/10'

Storage: 4°C on infinite setting (until removal).

2.4.3.4 PCR four:

Cytb primers as per PCR three.

PCR master mix per sample:

Master mix 2x (Promega) 12.5 µl
Forward primer (diluted 1:10 original stock solution: Nuclease-free water) 2.5 µl
Reverse primer (diluted 1:10 original stock solution: Nuclease-free water) 2.5 µl
Template DNA 5 µl
Nuclease-free water 2.5µl

PCR programming (Forgham *et al*., 2015):

Initial denaturation: 94°C/5'
Denaturation: 94°C/1'
Annealing: 58°C/1'
Extension: 72°C/16" (35 cycles of denaturation, annealing, elongation)
Final extension: 72°C/7'
Storage: 4°C on infinite setting (until removal).

2.4.4 PCR check: Gel electrophoresis

DNA visualisation after PCR processing was conducted by use of agarose gel electrophoresis.

For each 1% gel, 50 ml Tris/Borate/EDTA (TBE) buffer solution and 0.5 g agarose (Fisher Scientific™), were placed into a conical flask and dissolved by microwaving on full power for approximately 50 seconds until no solid particles were visible in the liquid solution.

The solution was left to cool for five minutes before adding 3 µl SafeView™ Nucleic Acid Stain (NBS Biologicals®), mixing gently and adding to an electrophoresis gel tray (a unit put together with rubber supports at each end and placement a plastic well comb). This unit which was then left twenty minutes with comb in place to allow the liquid to cool and set to a gel consistency.

The outer rubber supports of the mould unit and comb were carefully removed. The gel was set inside the electrophoresis unit (Fisher Scientific™) and filled to maximum level with TBE x1 liquid.

(Note: the TBE was directly poured over the wells to ensure any contaminants such as sodium deposits were washed from the wells.)

For each sample, 1 µl 6x blue/orange loading dye (Promega) and 5µl sample pipetted into a new clean 1.5 ml Eppendorf, pulse- vortexed and briefly centrifuged (no greater than five seconds). A 100 bp DNA Ladder (Promega) ladder was also mixed in this way (5 µl ladder: 1 µl dye).

The 6 µl of each sample and dye mix was loaded into each well of the 1% gel. The DNA ladder was loaded into the first well followed by each sample and a negative control sample in the final well.

Electrophoresis was run for 50 minutes at 100v once all samples were loaded.

To assist in sample choice when trialling different PCR methods, 5 µl of extracted samples with 2 µl dye per sample were directly run through gel electrophoresis in order to identify DNA bands.

Following PCR, gels were checked for detectable bands by placing into the tray of the Bio-Rad™ Molecular Imager, and running the Gel Doc™ XR+ Image Lab™ Software 5.2.1, selecting Nucleic Acid Gels; SYBR Safe; Faints Bands for detection, to capture images of each gel.

2.5 Analytical Methods: Sequencing

Sequencing was chosen as the data analysis process to validate those samples amplified, and to evidence the effectiveness of the field methods for viable genetic material.

2.5.1 Preparing PCR samples

Post-PCR clean-up was conducted to remove any potential post-cycle sequencing reaction contaminants from the amplified DNA that may interfere with sequencing results. The clean-up was conducted as per protocol Wizard® SV Gel and PCR Clean-Up System: Quick Protocol (Promega, 2011) with the following adjustments:

Gel Slice and PCR Product Preparation:

Preparation: Protocol followed from Step B with volume adjusted according to PCR amplification quantity.

Storage: Remains at -20°C until required. The complete protocol is provided in Appendix VII.

2.5.2 Sequencing

Following the clean-up protocol, amplified samples are posted to the London, UK department of a German company GATC Biotech®. Preparation of samples to be posted was conducted as follows:

5 µl PCR product (20-80 ng/µl DNA content optimum) was pipetted into a sterile 1.5 ml Eppendorf.

2.5 µl Forward primer (1:10 as per original PCR mix), and 2.5 µl nuclease-free water was added.

The Eppendorf was sealed securely (without parafilm) and labelled with Biotech barcodes that register to the University of South Wales.

A second sequencing method replaced the forward primer with the reverse (1:10 as per original PCR mix), and included a positive control.

Upon return of results, nucleotide sequences of each sample were entered into the Basic Local Alignment Search Tool (BLAST® 2.6.1**)** system of the National Center for Biotechnology Information® (NCBI®). The program available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi compares sequences of nucleotides and proteins of sample to those entered on the software database. Statistical significance of results was identified by identifying biological regions of similarity, using a scoring system against an expected (E) value and similarity percentage to assess the biological relevance of results. Those samples that produced similarities scores of 90% or less were disregarded when *cytb* primers were part of the sample mix. This is due to the universal primers' ability to detect multiple species, of which can gain multiple alignment matches but often non-accurate results. Primer sequences, used in sequencing were also entered in The BLAST software to test for primer suitability, and report any species bias (NCBI, 2017). When similarity was greater than 90%, the identified species were entered into the Primer Blast system to record the number of nucleotide mismatches in the four most common sequences. The target species was also entered for comparison.

Chapter 3. Results

3.1 Lion Biological Sample Comparison

Lion samples were used to provide the genetic comparison of DNA quality and quantity obtained from different sample types.

3.1.1 Lion sample DNA quantification

DNA extracted from hair samples LNH01 and LNH02, followed by tissue sample LNT03 produced the highest quantities of DNA at levels of: 489.6 ng/µl; 259.9 ng/µl, and 97.7 ng/µl, respectively (fig. 3.1). Tissue sample LNT01 and faecal sample LNF02 produced the lowest quantities of DNA with results of 6.9 ng/µl and 11.0 ng/µl (fig 3.1). All samples produced a DNA quantification of more than 5 ng/µl (table 1).

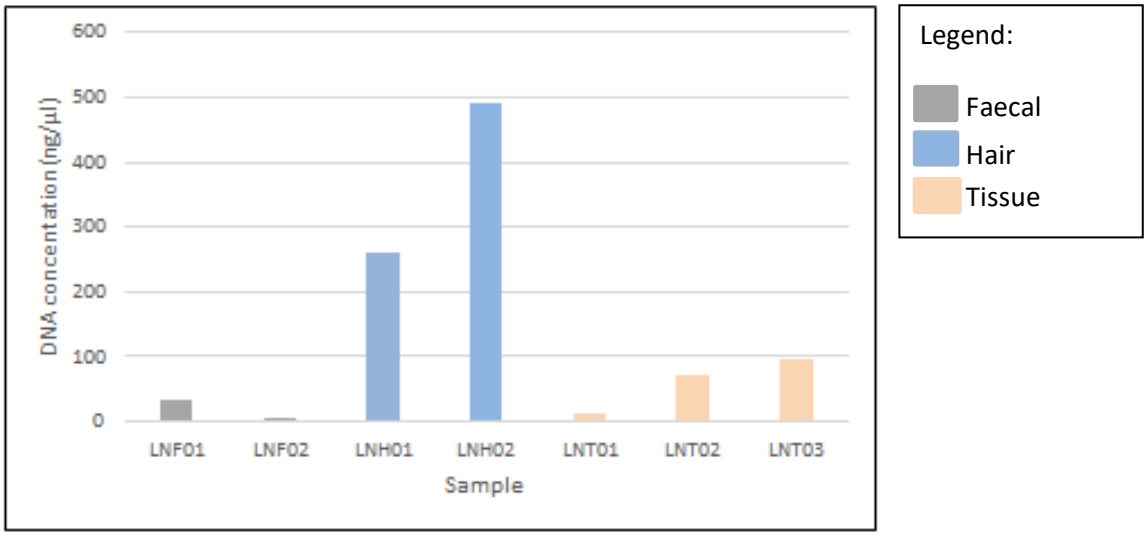

Figure 3.1. DNA concentration of purified and extracted African lion (*Panthera leo*) samples to provide a quantitative comparison between faecal, hair and tissue biological samples. From three applied preservation methods, faecal sample used stored by ethanol and frozen.

The purest DNA samples upon primary analysis (260/280) were the hair sample LNH01 and tissue sample LNT01 at values of 2.0 and 1.96 respectively. The purest sample upon secondary analysis (260/230) was hair sample LNH01 at a value of 1.75 ng/µl. Hair sample LNH02 and tissue samples

LNT01 and LNT02 produced the lowest 260/280 purity values at 0.49 ng/µl, 1.4 ng/µl and 1.43 ng/µl respectively (table 1).

Both faecal samples LNF01 and LNF02 produced 260/280 ratios of greater than 2.0, indicating the potential of RNA within the extracted sample. These samples also indicated the lowest secondary purity ratios of 0.17 (LNF02) and 0.18 (LNF01).

Table 1. DNA purity values of different African lion (*Panthera leo*) sample types. Table includes sample storage, extraction and PCR methodology.

Sample	Sample Type	Storage Method	Sample Size	Extraction Protocol	PCR Protocol	Concentration (ng/ µl)	260/280 (primary purity value)	260/230 (secondary purity reading)
LNT01	Tissue	Ethanol	0.014mg	1	1, 2, 3, 4	11.0	1.4	0.35
LNT02	Tissue	Ethanol	0.014mg	1	1, 2, 3, 4	71.6	1.43	0.61
LNT03	Tissue	Ethanol	0.014mg	1	1, 2, 3, 4	97.7	1.96	0.59
LNF01	Faecal	Ethanol & Dry	0.079mg	2	1, 2, 3, 4	33.1	2.29	0.18
LNF02	Faecal	Ethanol & Dry	0.185mg	2	1, 2, 3, 4	6.9	4.08	0.17
LNH01	Hair	Dry: Falcon tube	6 hairs	3	1, 2, 3, 4,	259.9	2.0	1.75
LNH02	Hair	Dry: Falcon tube	10 hairs	3	1, 2, 3, 4	489.6	0.49	0.21

Lion hair, tissue and faecal samples directly loaded onto an electrophoresis gel revealed only the hair samples were visible, thus indicating substantial DNA content. DNA content of faecal and tissue samples are not visible indicating DNA content lower than 20 µl/ng (fig. 3.2).

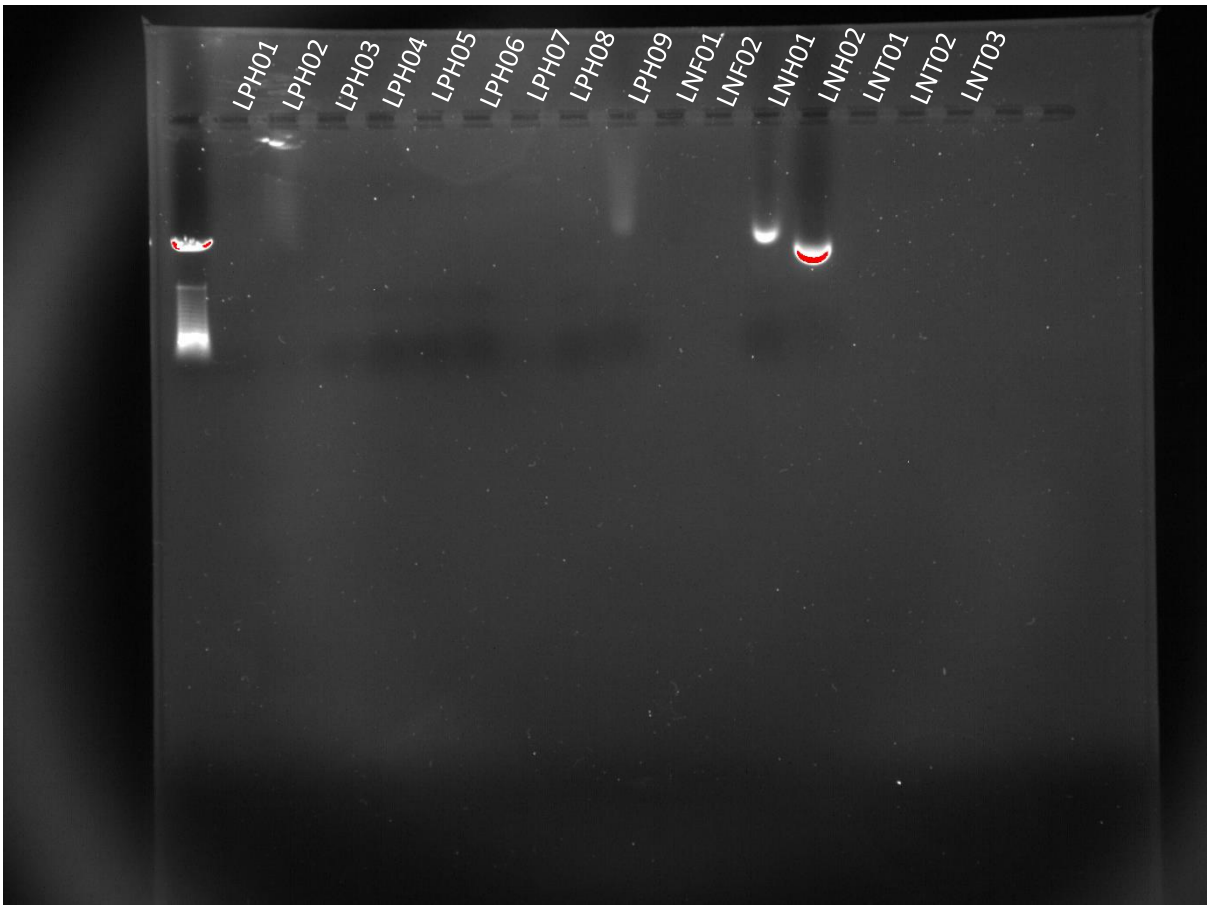

Figure 3.2. Extracted samples loaded directly into 1% agarose gel to assist in sample choice for PCR trials. Electrophoresis identified strong DNA bands in samples LNH01 and LNH02.

3.1.2 Lion sample DNA amplification

No tissue samples (LNT01; LNT02; LNT03), or faecal samples (LNF01, LNF02) were successfully amplified on any PCR protocol. Directly-loaded DNA of these samples also did not show upon electrophoresis indicating DNA content is below 20 ng/µl.

Gel electrophoresis identified amplified bands of sample LNH01 on PCR protocols one (fig. 3.3), two (fig. 3.4) and three (fig. 3.7: Leopard sample DNA amplification section). Sample LNH02 was amplified in PCR protocol two (fig. 3.4) and PCR protocol four (fig. 3.9; fig. 3.10; fig. 3.11). Both lion hair samples were applied as a later positive control, and are therefore presented in leopard sample DNA amplification 3.2.2 section.

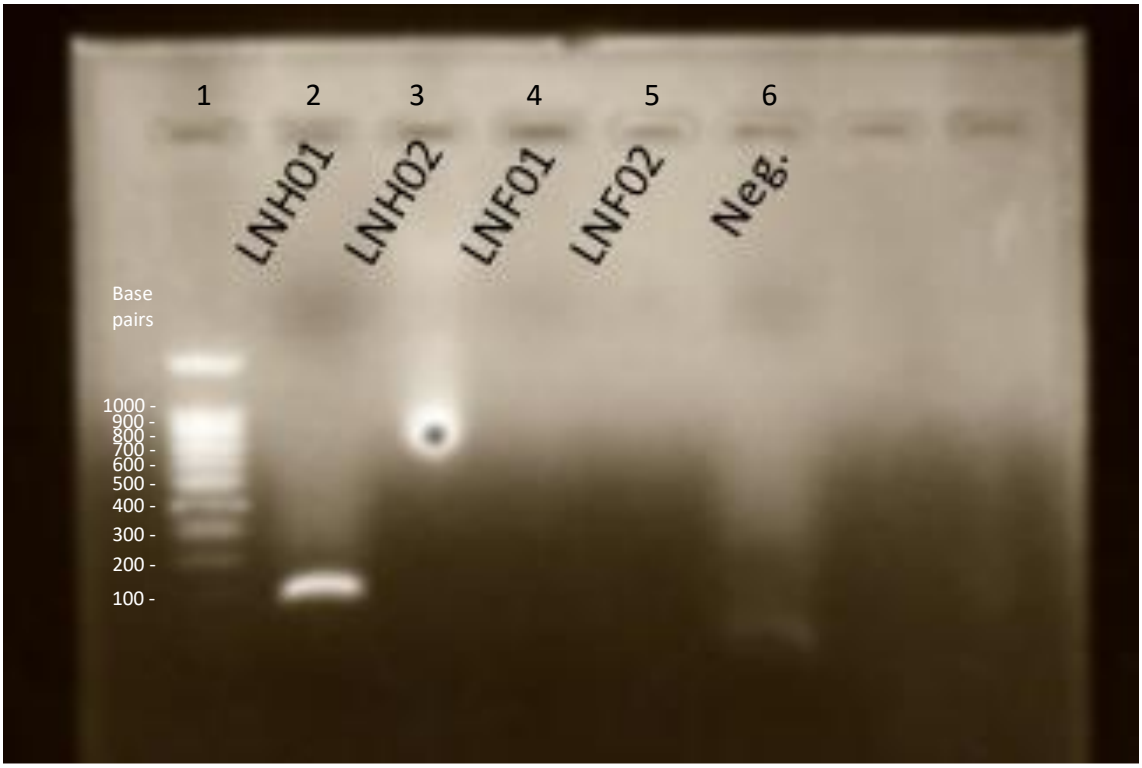

Figure 3.3. African lion (*Panthera leo*) hair and faecal samples with negative PCR control on a 1% agarose electrophoresis gel. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol one applied targeting 126 bp DNA band within the mitochondrial *ATP6* gene. Sample LNH01 DNA band is visible in lane two.

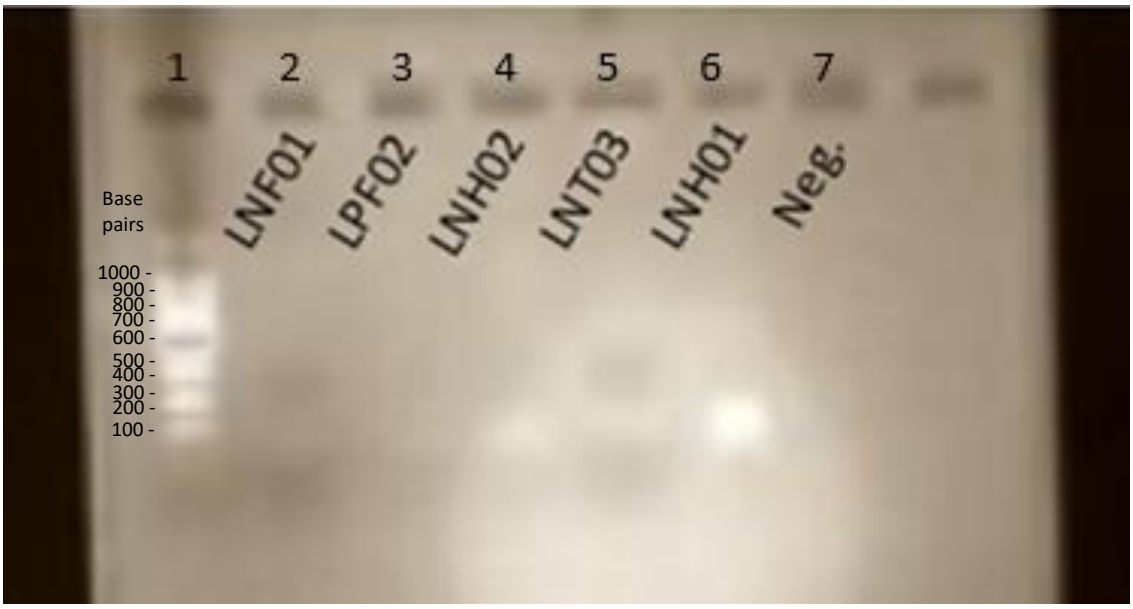

Figure 3.4. African lion (*Panthera leo*) hair, faecal and tissue samples with negative PCR control on a 1% agarose electrophoresis gel. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol two applied targeting 187 bp DNA band within the mitochondrial *COI* gene. Sample LNH02 DNA band is visible in lane four and LNH01 in lane six.

3.1.3 Lion sample sequencing

Following successful amplification and clean-up protocols, the samples were again measured for concentration and purity values. Both lion samples LNH01 and LNH02 measured within the optimal concentration range of 20-80 ng/µl for sequencing (table 2).

Table 2. African ion (*Panthera leo*) hair samples quantified following PCR.

Sample	DNA Concentration (ng/µl)	260/280 (primary purity value)	260/230 (secondary purity reading)
LNH01	31.1	1.51	-0.27
LNH02	21.2	1.42	-0.25

Upon return of Biotech results, both sequences were too short for species identification. There was no significant similarity between sample sequence and any species on the NBCI Blast® biological sequence database (table 3).

Table 3. African lion (*Panthera leo*) hair sample sequencing by GATC Biotech.

Sample	Sequence	Result
LPH01	No result	Non-significant
LPH02	No result	Non-significant

To summarise these trials, amplification occurred for both lion hair samples but no positive sequencing result was produced. Therefore, no species alignment match was reported.

3.2 Leopard Non-Invasive Sampling

3.2.1 Leopard bait site presence

Leopard presence and associated sample collection occurred across six of the 16 bait sites (figure 3.5). Camera trap evidence data indicated the presence of seven leopards; with distinguishing features evidenced in fig. 3.6 (full camera trap photographs are available in appendix IX). Proposed individual L7 could not be identified as being either a new or existing individual through poor quality visual data. All other camera trap data allowed accurate interpretation. Two individuals, L1 and L2 were present at bait trap 1 during the same time period with samples collected from both individuals. Individuals L4 and L5 were both present at bait site 5, but only L5 made contact with the bait site mechanism, and so one sample was collected from this site. L3 occurred at both bait site 3 and bait site 4.

Table 4 represents a first glance at those individuals whose sample(s) were amplified. Section 3.2.2 goes on to look into the quantity and quality of the samples that were trialled. Hair samples collected from bait site 4 were not successfully amplified; however, the individual L3 occurred at both bait sites 2 and 4 with the latter bait site showing successful amplification.

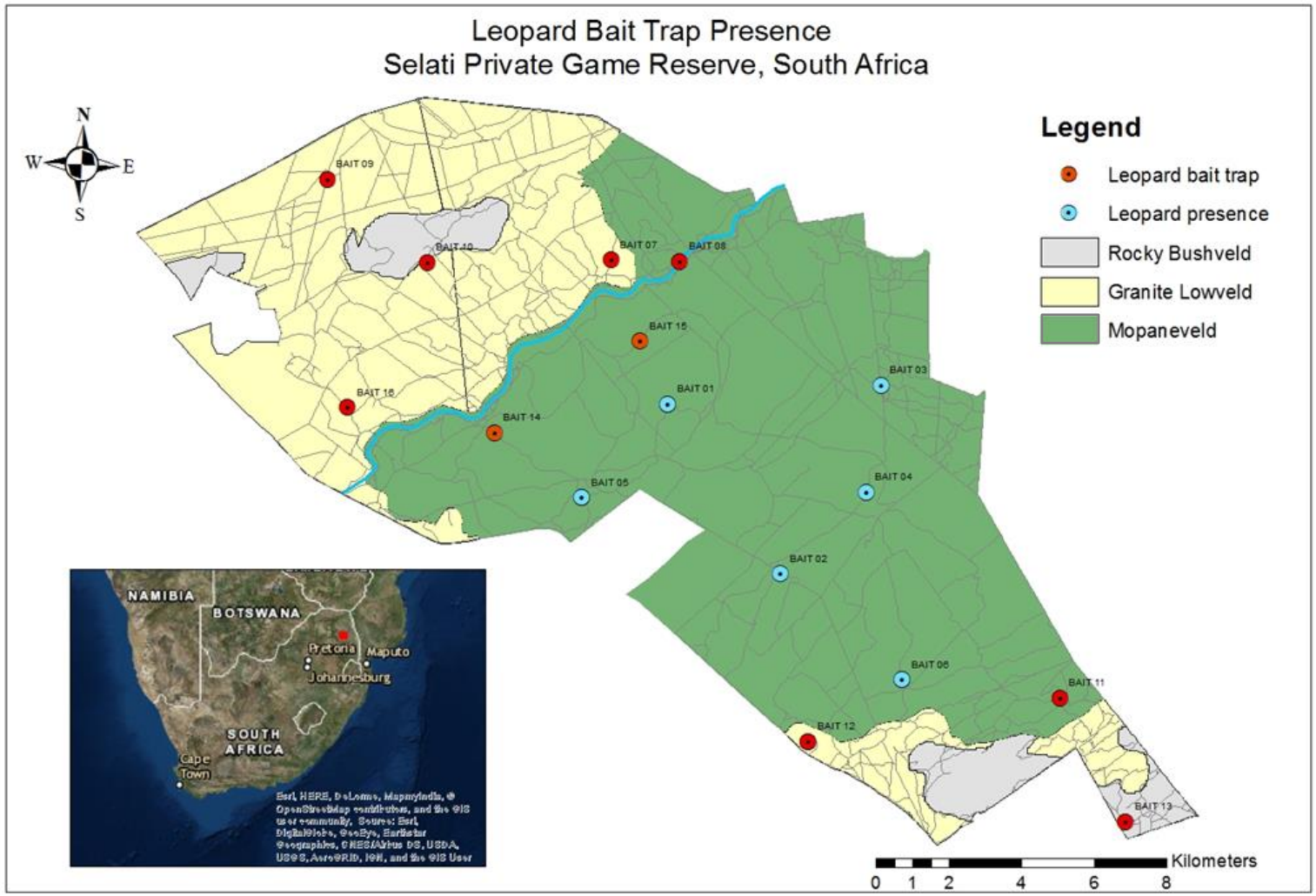

Figure 3.5. African leopard (*Panthera pardus pardus*) presence occurred at six of 16 bait traps across the 29, 411 ha Selati Game Reserve, South Africa. Hair samples were collected at all sites in which presence was recorded. Leopards were only detected in Mopaniveld habitat type.

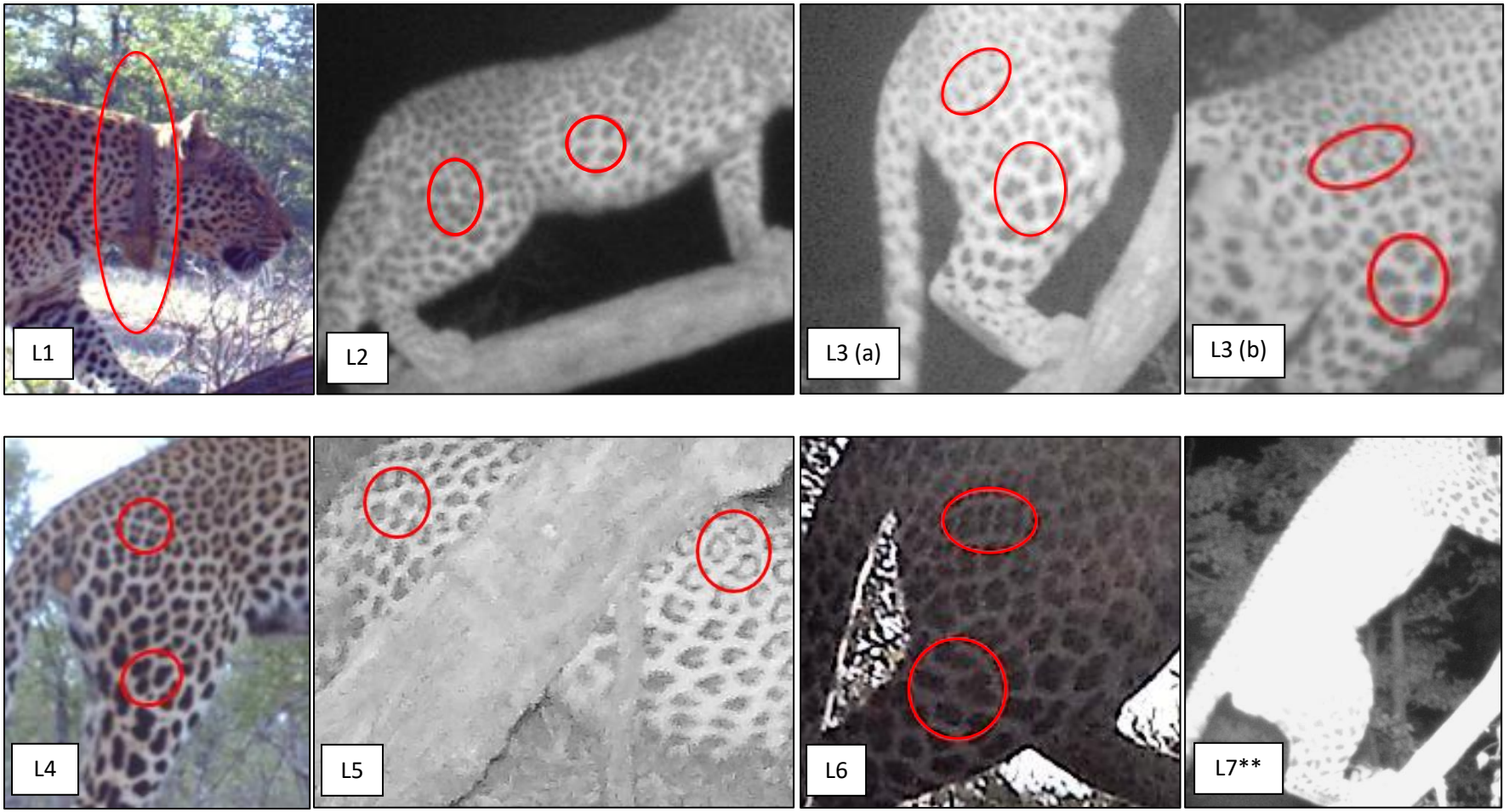

Figure 3.6. Cropped images from bait site camera trap data display the distinguishing features used in individual African leopard (*Panthera pardus pardus)* identification. Images are labelled by the code assigned to each leopard individual. L3 (a & b) show the same individual from two bait trap locations, ** represents a proposed L7 that could not be confidently identified as a new/existing individual from camera trap data. Complete images and details of bait trap locations are available in appendix VI.

Table 4. African leopard (*Panthera pardus pardus*) sample collection and genetic application.

Bait Trap Number	Location	No. of Leopards	Species Interference *	Individual ID	Samples Collected (Y/N)	Amplified (Y/N)	Sequenced (Y/N)
1	Wildebeest Way	2	Honey Badger	L1	Y	Y	N
				L2	Y	Y	N
2	Thankerton Cutline	1	African Civet	L3	Y	Y	N
3	Steve's Cutline	1	African Civet	L4	Y	Y	N
4	Burren Koppie	1	-	L3	Y	N	N
5	Jacket Plum	2	-	L5	Y	Y	N
			-	L6	N	-	-
6	Salejan	1	-	L7**	Y	Y	N
7	Missi Link	0	-	-	-	-	-
8	South River Main	0	-	-	-	-	-
9	Lillie Dam Cutline	0	-	-	-	-	-
10	The Deck	0	-	-	-	-	-
11	Boulders	0	-	-	-	-	-
12	Southern Fenceline	0	-	-	-	-	-
13	Lillie II	0	-	-	-	-	-
14	Concrete Crossing	0	-	-	-	-	-
15	Oxwagon North	0	-	-	-	-	-
16	Bataleur	0	-	-	-	-	-
	Total	8		7	7	6	

* represents bait trap interference by mammalian species other than leopards.

** represents a proposed L7 that could not be identified as a new/existing individual by camera trap data.

3.2.2 Leopard sample DNA quantification

As recorded in table 5, samples LPH04, LPH09 and LPH13 had the greatest concentrations of DNA with 401.5 ng/µl, 26 µl and 26 µl respectively. The lowest DNA quantities were extracted from samples LPH11 and LPH15 with minus values signifying non-successful extraction. The purest samples were LPH01 and LPH03 upon primary analysis with 260/280 ratio values of 1.81 and 1.89 respectively. The purity of LPH01 was supported by secondary analysis with a highest 260/230 ratio value of 1.51, however, this was not the case for sample LPH03 with a 260/230 ratio of -0.28.

Samples LPH05, LPH08 and LPH10 produced 260/280 ratios of greater than 2.0, indicating the potential of RNA within the extracted sample.

Table 5. DNA extraction results of African leopard (*Panthera pardus pardus)* hair samples and an overview of amplification and sequencing success.

Sample	Individual	Hair Storage Type	Sample Size (no. hairs)	Extraction Protocol	PCR Protocol	DNA Concentration (ng/µl)	260/280 (primary purity value)	260/230 (secondary purity reading)	Amplified (Y/N)	Sequenced (Y/N)	Sequenced Species Match
LPH01	L1	Dry	10	3	1 ,4	2.5	1.81	1.54	Y	Y	African civet
LPH02	L2	Dry	10	3	2, 4	2.5	1.64	0.33	Y	N	-
LPH03	L3	Dry	20	3	4	1	1.89	-0.28	Y	Y	Bovine
LPH04	L4	Ethanol	6	3	1, 4	401.5	0.88	0.59	Y	N	-
LPH05	L2	Ethanol	6	3	3, 4	1.3	3.62	5.95	Y	N	-
LPH06	L3	Ethanol	10	3	2,4	3.6	1.74	0.31	N	N	-
LPH07	L6	Dry	20	4	4	2.7	-1.79	1.06	Y	N	-
LPH08	L7	Dry	10	4	1, 3, 4	1	11.24	0.26	N	N	-
LPH09	L4	Dry	12	4	1 ,3 ,4	26	-0.04	0.94	Y	N	-
LPH10	L2	Dry	12	4	4	1.2	5.05	1.15	Y	N	-
LPH11	L1	Ethanol	1	4	4	0*	-	-	N	N	-
LPH12	L5	Dry	20	4	4	3.4	1.22	0.94	Y	N	-
LPH13	L6	Dry	4	4	4	26	1.42	0.63	Y	N	-
LPH14	L3	Dry	30	4	4	6.1	1.06	0.94	N	N	-
LPH15	L3	Dry	10	4	4	0*	-	-	N	N	-
Total	7	-	-	-		-	-	-	10	2	-

*Minus values have been recorded as zero to indicate non-successful extraction of DNA.

3.2.3 Leopard sample DNA amplification

A total of ten of the fifteen leopard hair samples were successfully amplified by application of PCR protocol 4. These samples all consisted of a concentration of 1 ng/µl or greater. Gel electrophoresis identified successfully amplified bands of leopard hair samples only by PCR protocols three and four, both of which use *cytb* primers.

Gel electrophoresis identified an amplified DNA band of sample LPH05 by protocol three (universal *cytb* primers) but with potential contamination evident in the negative control (fig. 3.7). The sample was re-tested and successfully amplified by PCR protocol four (the identical *cytb* primers with different PCR conditions) along with samples LPH01; LPH02; LPH03 and LPH04. Sample LPH08, loaded in the same gel did not amplify (fig. 3.8). Other samples amplified by PCR protocol four were LPH13 (fig. 3.9); LPH09; LPH10 (fig. 3.10) and LPH07 (fig. 3.11).

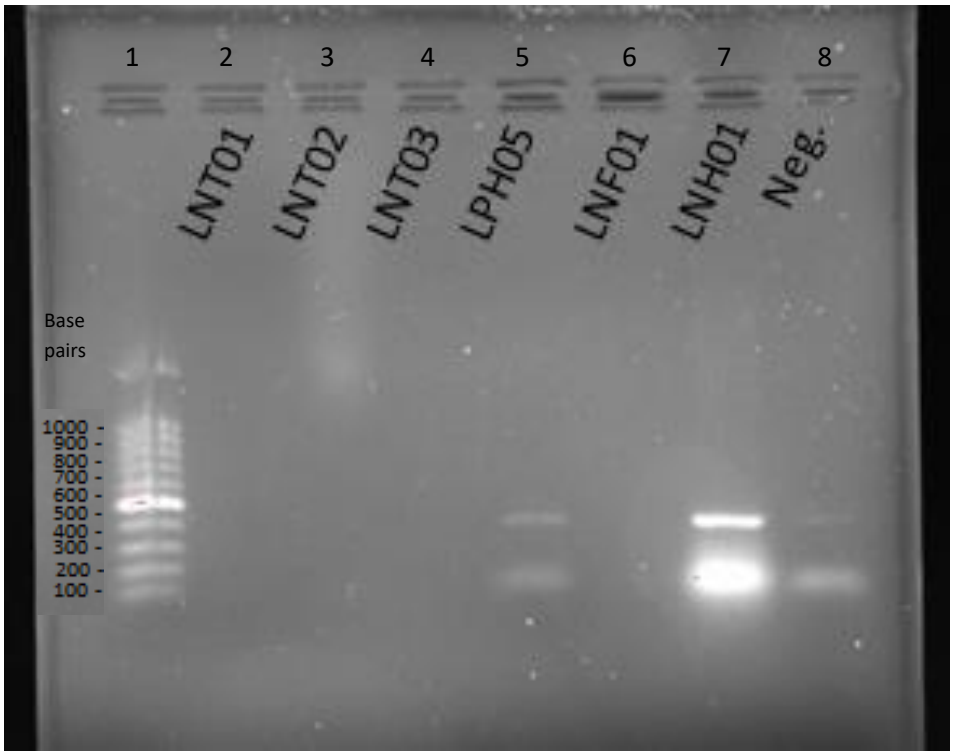

Figure 3.7. African leopard (*Panthera pardus pardus*) hair samples with negative PCR control on a 1% agarose electrophoresis gel. Sample LNH01 provides a positive control. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol three applied targeting 358 bp DNA band within the mitochondrial *cytochrome b* gene. Sample LPH05 DNA band is visible in lane five and LNH01 in lane seven. Negative control shows evidence of contamination (lane 8).

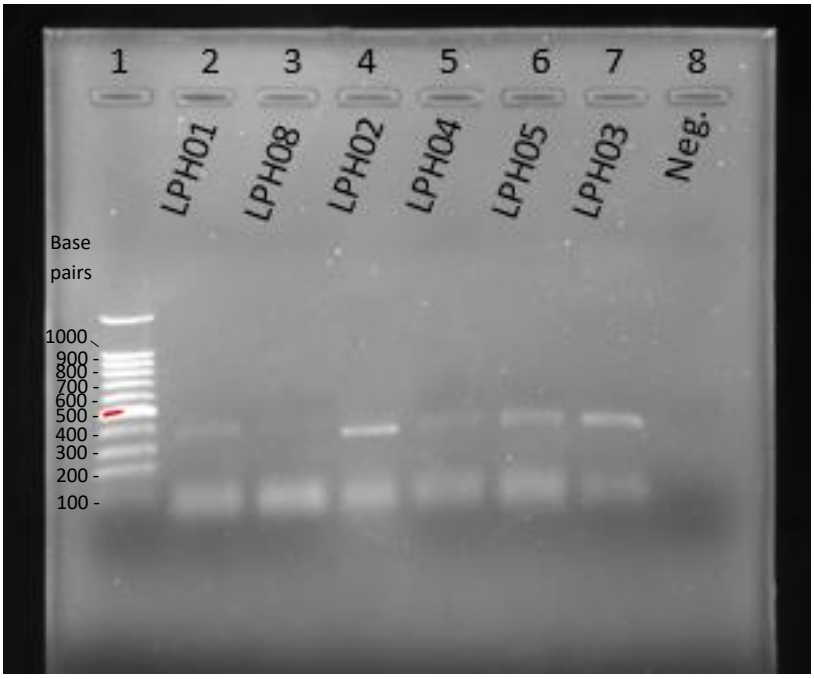

Figure 3.8. African leopard (*Panthera pardus pardus*) hair samples with negative PCR control on a 1% agarose electrophoresis gel. Sample LNH01 provides a positive control. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol four applied targeting 358 bp DNA band within the mitochondrial *cytochrome b* gene. DNA bands are visible for samples: LPH01; LPH02; LPH03; LPH04 and LPH05. Smears at the base of lanes two to seven represent primers. Negative control (lane 8) consists of nuclease-free water used in the PCR mix and 6x loading dye.

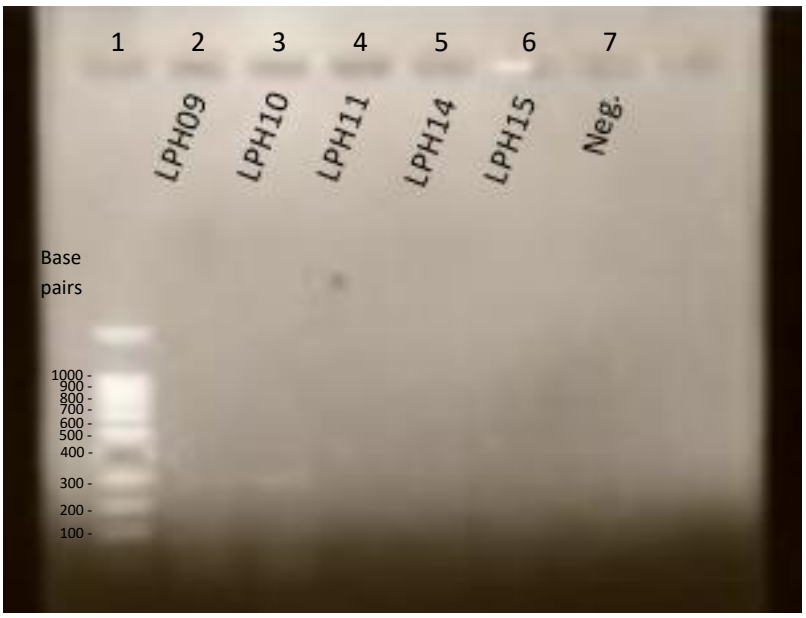

Figure 3.9. African leopard (*Panthera pardus pardus*) hair samples with negative PCR control on a 1% agarose electrophoresis gel. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol four applied targeting 358 bp DNA band within the mitochondrial cytochrome b gene. Faint DNA bands are visible for samples: LPH09 (lane two) and LPH10 (lane three). Smears at the base of wells represent primers. Negative control (well 7) consists of nuclease-free water used in the PCR mix and 6x loading dye.

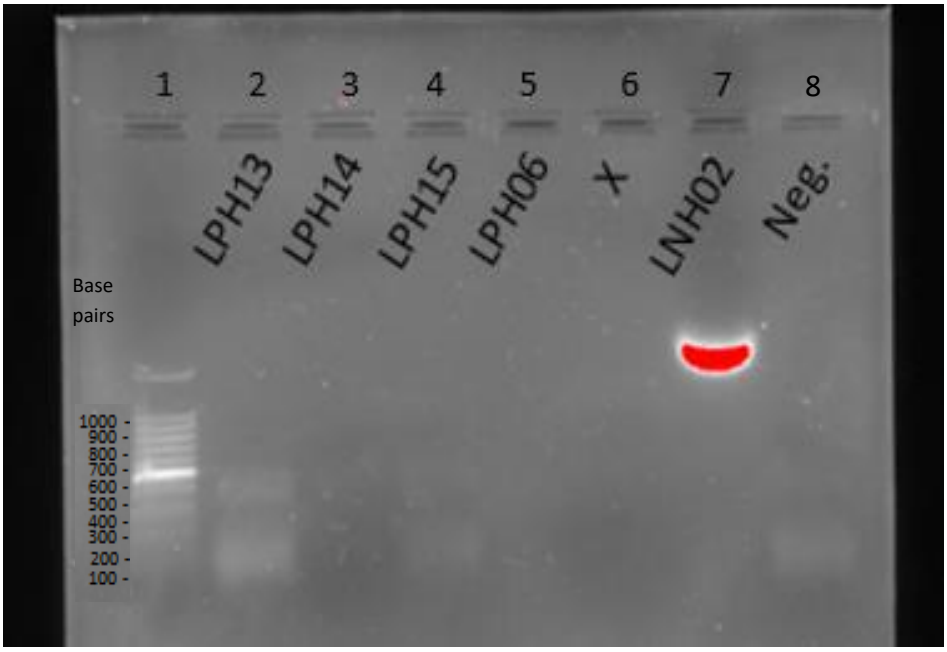

Figure 3.10. African leopard (*Panthera pardus pardus*) hair samples with negative PCR control on a 1% agarose electrophoresis gel. Sample LNH02 provides a positive control. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol four applied targeting 358 bp DNA band within the mitochondrial *cytochrome b* gene. A DNA band is visible for sample LPH13 (lane two). LNH02 is applied as a positive PCR control. Smears at the base of lanes two, four and eight represent primers.

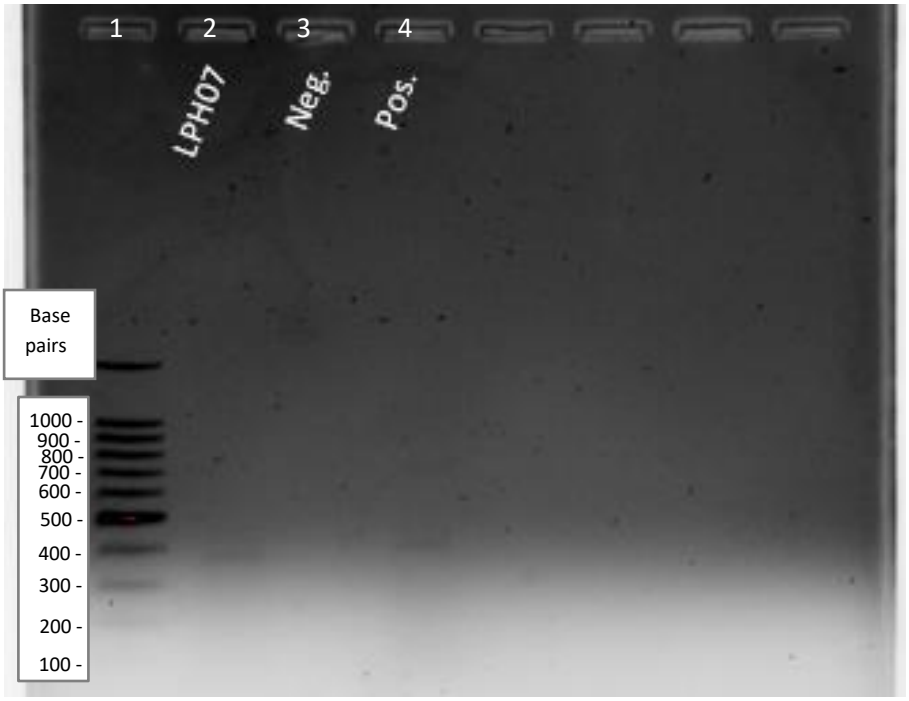

Figure 3.11. African leopard (*Panthera pardus pardus*) hair samples with negative PCR control on a 1% agarose electrophoresis gel. Sample LNH02 provides a positive control. Lane one consists of 100 base pair (bp) DNA ladder (see labels for sizing). PCR protocol four applied targeting 358 bp DNA band within the mitochondrial *cytochrome b* gene. A faint DNA bands is visible for sample LPH07 (lane 2). Contrast was altered for improved visualisation.

3.2.4 Leopard sample sequencing

The DNA concentrations of each sample sent to Biotech for sequencing are recorded in table 6. Only LPH03 provided a statistically significant similarity between database and sample sequence in the first sequencing procedure. In the second sequencing trial LPH03 was included as a positive control, and sample LPH09 which falls below the optimal (20-80 ng/µl) is excluded.

Table 6. African Leopard (*Panthera pardus pardus*) hair samples quantified following PCR and clean up protocol.

Sample	DNA Concentration (ng/µl)	260/280 (primary purity value)	260/230 (secondary purity reading)
LPH01	73.9	1.33	-4.12
LPH02	27	1.47	-0.22
LPH03	43.4	1.63	-0.53
LPH04	53.7	1.65	-0.87
LPH05	43.9	1.66	-0.61
LPH07	49.8	1.58	-0.64
LPH08	28.0	1.83	1.09
LPH09	13.1	1.3	-0.30
LPH10	24.7	1.8	-6.03
LPH12	32.1	1.9	1.63
LPH13	55.5	1.54	-0.68

Upon return of initial results, only LPH03 sample sequence was at sufficient base pair length for detection of species similarity (table 7).

Table 7. GATC Biotech Initial sequencing results. Samples amplified with PCR primer *cytochrome b*.

Sample	Sequence	Sequence Length (bp)	Species Result
LPH03	tnacAanTCcTAACAGGgCTATTCTTAGCCATACACTACACAgcag aCACAACAACCGCCTTCTCATCAGTCACCCACATTtGTCgngatg TTAATTATGGCTGAAttATCCGATACATACACGCCAATGGAGCT TCCATATTTTTTAttnGCTTATTCATTCATGTAGgcnGAGgAaTAT ACTATGGTTCCTATACCTTCTCAGAAACATGAAATATCGGAATT CTATTattattCGCAACCATAGCCACAGCCTTCATAGGCTAcGTCC TACCATGAGGACAAATATCATTCTGAGGGGca	301	*Civettictis civetta*

Species similarity result concluded significant similarity with the African Civet (*Civettictis civetta*) shown in fig. 3.12 (Bit-score 529 (286), similarity = 98%, E= 2e-146).

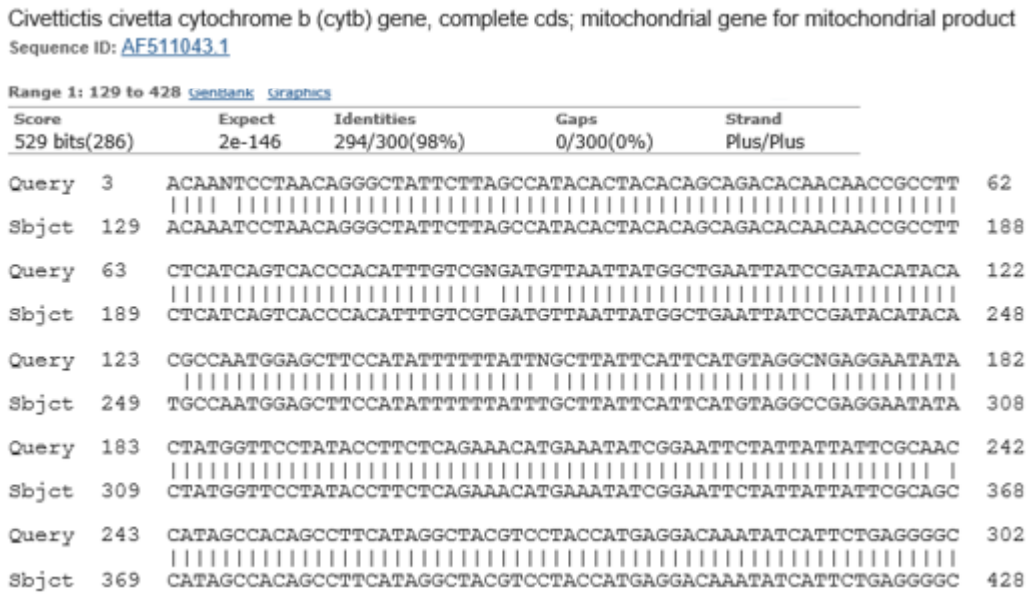

Figure 3.12. Sequence alignment match identified similarity of 98% for African civet (*Civettictis civetta*) *cytochrome b* gene, complete coding sequence; mitochondrial gene.

Species sequences similarities were identified for samples: LPH01; LPH02; LPH03; LPH07; LPH08, LPH10, LPH12 and LPH13. Two of these results produced a significant similarity match with species from the biological database (samples LPH01 and LPH03), whilst others could not gain a conclusive (i.e. greater than 90% similarity) match. Samples LPH04 and LPH05 did not produce a result and so were not entered into the sequence database search.

Table 8. GATC Biotech second sequencing results. Samples amplified with *cytochrome b* primers.

Sample	Sequence	Sequence length (bp)	Species Result
LPH01	tggtGTGAGcAGaAGGATtaCTCcnntatTTCatGTTTCtnnanaaGTGtan gAcCCGTAATATAagCctCGTCCtAcGtg	81	*Bos taurus*
LPH02	ATtaCTCcnntattTCATGTTTCtanaaaAgtGtAngAnCCGTAATATAanC CTCGTCctangtGcATaTAtnAgcngAtnAanAnnantga	92	No significant result
LPH03	TtCCGATATTTCATGTTTCTGAgAAGGtgaTAGGAACCATAGTATATTC CTCGGCCTAcatGAATGAATAAGCAAATAAAAAATATGGAAGCTCCA TTGGCGTGTATGTATCGGATAATTCAGCCATAATTAACATCACGACA AATGTGGGTGACTGATGAGAAGGCGGTTGTTGTGTCTgctGTGTAGT GTATGGCTAAGAATAgCccTGTTAGGATTTGTAAaaTAAGGCAGATTC CTAGTAGGGAGCCGAAATTTCATCATGCTGAGatGTTGGATGga	282	*Civettictis civetta*
LPH04	No result	0	No significant result
LPH05	No result	0	No significant result
LPH07	TCCtgTgtTTCagGTTTCTnnannnnGtAcGAnccntaaTATAgGCCgCGtC CtatGTgtnngaagAtGCAgATgAAgAanAntGagGcgccA	93	No significant result
LPH08	GtacgAnCCgTAATATAgGccgCGTCCnatGTGtanGAagAtGcagATgAa gAanAntGAgGcgcCATTnnnntGnagGTTaCgGAtgAntCaACCGTAnt	101	No significant result
LPH10	GATTaCtCcnnTattTCATGTTtctnnnnnnnnnGnaatgAnccGTAATATAag CCtCGTcctangtg	67	No significant result
LPH12	gAcCcgTAATATAancCtCGtCCtangtgtananatnAngnagATnaaana	51	No significant result
LPH13	AtgtGgGccAcagAngAgAATGCtGntgttgtGTCnGAtgtGtaatGtaTtGct AggAATAggcctGtGAnGAtTTGnagGAtTAgGC	-	No significant result

Sequencing of sample LPH01 identified similarity with bovine (*Bos taurus isolate*) shown in fig. 3.13 (Bit-score 122 (66), similarity = 92%, E= 6e-25).

Sample LPH03, processed as a positive control in the second sequencing trial again identified African civet (*Civettictis civetta*) shown in fig. 3.14 (Bit-score 492 (266), similarity = 98%, E= 2e-135) as a match.

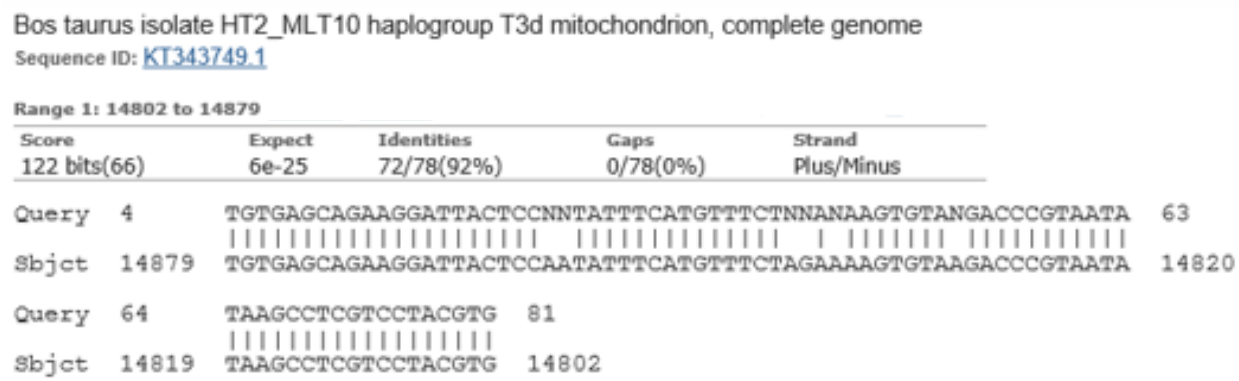

Figure 3.13. Sequence alignment identified 92% similarity for bovine (*Bos taurus isolate*) mitochondrion, complete genome.

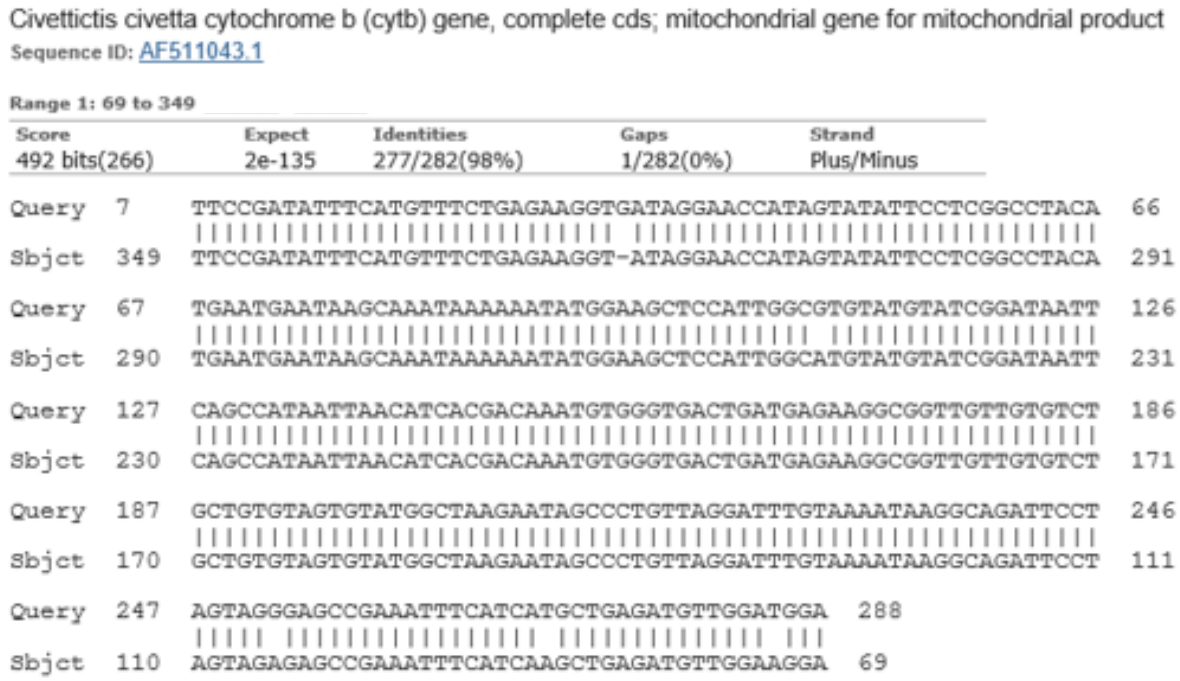

Figure 3.14. Sequence alignment match identified similarity of 98% for African civet (*Civettictis civetta*) *cytochrome b* gene, complete coding sequence; mitochondrial gene.

As amplifications only occurred when using *cytb* primers, the forward and reverse sequences were checked for suitability to the target species. Comparison of the primer sequences to database sequences of the two significant matches revealed both had a lower number of mismatches (*Civettictis civetta,* taxid 9691: four mismatches; *Bos Taurus,* taxid 9913: two mismatches) than the target species (*Panthera pardus*, taxid 9688: five mismatches).

Chapter 4. Discussion

4.1 Biological Sample Comparisons

In this study's comparison of three biological sample types, hair samples produced the highest concentrations of extracted DNA followed by tissue, and faecal produced the lowest. Hair and tissue also produced the purest qualities of DNA in primary ratio interpretations, with hair sample LNH01 showing the optimal 2.0 ratio, followed closely by LNT03 at 1.96. Sample LNH01 again produced the purest quality in secondary ratio interpretation and in comparison, the remaining samples all produced low purity values. Such findings support both hair and tissue as sample choices in wildlife research by producing high DNA quantity and quality. Regarding common sample problems recognised by Taberlet *et al*. (1999), such readings may indicate minimal sample and DNA deterioration of hair and tissue. This may be through adequate preservation and extraction techniques applied to the appropriate biological sample type, thus resulting in high quality DNA. Past research into Northern (Sloane *et al.,* 2000) and Southern (Walker *et al.,* 2006) hairy-nosed wombats demonstrated that applying the most suitable preservation techniques and minimising storage time before extraction resulted in high quality useable DNA with little loss sample loss. As both conducted research based on hair sampling strategies using adhesive mechanisms, this consequently shows potential for this and similar studies if sample quality remains high after collection.

Hair was the only sample type that produced DNA bands, indicating successful amplification of the MtDNA target sequence. Four PCR protocols were applied to all sample types and each produced valid bands thus supporting the usability of the DNA with regards to various PCR conditions. As such, hair samples were later used as a positive control in trialling PCR conditions for leopard hair sample amplification. In considering that sample LNH02 did not have optimal DNA purity, its amplification and usefulness in this study further demonstrates that hair as a type of biological sample, rather than individually 'pure' samples, is effective in producing useable DNA.

Although tissue samples of this study produced lower concentrations and quality of DNA, findings do support a high quality source of DNA when considered with high primary purity readings and past literature. It has been indicated that tissue samples were regarded as the highest quality DNA for genetic wildlife research with particular areas of complexity in soft tissue cells out-performing those of non-invasive alternatives (Iverson *et al*., 2004; McFadden *et al*., 2006; Thiemann *et al*., 2006; Roberts & Podesta, 2015). However, as Roberts & Podesta (2015) and Taberlet *et al.* (1999) inferred this may largely be due to the level of non-invasive molecular interpretation and subsequent

downstream genetic application not yet explored to a comparable extent. Despite this, research by Wasser *et al.* (1997) genotyping *Ursid* individuals using different sample types showed no difference when DNA was extracted from blood and tissue compared to faecal matter, although excretions were collected and stored within six hours, a practice that is not always possible in field collection. Nevertheless, with the increasing use and successful outputs recognised by a variety of wildlife research and the outputs of this study, the potential of non-invasive genetics should not be dismissed.

Faecal samples of this study produced the poorest concentrations and quality of DNA when compared to hair and tissue. No amplifications resulted from PCR trials and therefore sequencing was unviable. Similar to the study by Wasser *et al.* (1997), excreted samples were fresh upon collection (less than one hour in field conditions) thus suggesting other factors may have hindered DNA usability. With regards to faecal analysis, previous literature has revealed varying degrees of success across a wide range of genetic investigations. Bhagavatula & Singh (2006) strongly supported the use of faecal sampling for obtaining molecular information, and produced accurate results in sequencing for species and population detection. Similarly, researching population dynamics by use of DNA from faeces has been accomplished by Wilson *et al*. (2003) and Prugh *et al*. (2005). Although previous studies reported success in terms of deciphering and interpreting outcomes back to the particular ecological question, reduction in sample size was often encountered in the laboratory. This may be through poor quality DNA where degradation or inhibitors limit usability, such as by Petit & Valiere (2006), or incorrect identification where sympatric carnivores were present as encountered by Bhagavatula & Singh (2006). This is a factor that could not be assessed and considered for this study, with such limited experimental sample size to begin with. Nevertheless, as a standalone investigation this study does effectively demonstrate those limitations that can be encountered in research relying solely on faecal sampling for genetic analysis.

As Taberlet *et al.* (1999) discussed, a shift in researcher priority from invasive, and historically destructive sampling to non-invasive alternatives was strengthened when non-invasive genetics was introduced as a practical option. However, following the advancement of using such techniques in the years prior to 1995; the persistent incidence of genotyping errors was reported by those researchers striving for efficient non-invasive genetics (Gerloff *et al*., 1995; Taberlet *et al*., 1996; Goossens*, et al*., 1998). Such technical complications might explain why after the initial and pursued development of these non-invasive methods, limited comprehensive studies have since been published and why these have largely been conducted to address the error risk itself. Detailed research by Wasser *et al*. (1997); Farrell *et al*. (2000); Piggot & Taylor (2003), and Penasco *et al*.

(2011) investigated the factors that can influence DNA degradation, and subsequent genotyping errors after field sample collection. It appears that sample processing techniques from field preservation to genetic application can influence outcomes of genetic analysis, regardless of choice between hair and faecal sampling. For this study, the storage methods appropriate to sample type and field conditions (semi-arid) according to past literature were applied with specific attention to the findings of Wasser *et al.* (1997); Taberlet *et al.* (1999); Bhagavatula & Singh (2006) and Panasco *et al.* (2011). With amplification only occurring in hair samples and no sequencing results, it is likely that success was hindered, particularly for the higher quality hair and tissue samples by limitations in sample processing. This may have been due to sub-optimal storage methods, contamination, human error or inadequate laboratory protocols.

Despite storage implications addressed, the findings of this study align with the results of others in that reasons for non-amplification and sequencing cannot be pinpointed with certainty. These findings agree with those inferences of Taberlet *et al.* (1999) that outcomes, or lack thereof may be due to inappropriate preservation choices. More rigorous and replicated studies using a variety of control conditions are required to thoroughly evaluate the performance of preservation protocols on different sample types, and on different species. As DNA sourced from tissue did not amplify, this leads to considerations into why PCR trials may have failed. Four PCR trials were applied which resulted in amplification of the same target species from hair, but not tissue despite high quality purity readings. When comparing tissue to hair, the impact of the lower DNA concentrations is evident when DNA samples were run directly onto an electrophoresis gel. The only samples that were visible were the hair samples, indicating that DNA concentration may be a major factor that affects PCR success. In addition, Penasci *et al.* (2011) expressed that inhibitory substances, can be an important limitation in the amplification stage of non-invasive samples, although faecal matter is more prone to such constraint. Another factor to consider is contamination, which is a possibility in any phase of handling or sample processing and can hinder sequencing performance (Waits & Paetkau, 2005). Aseptic techniques were used in this study, but the potential of contamination from solutions used from one step to the next can result in remnants of substances inhibiting extraction, amplification or sequencing. Further to this, human error can impact outcomes especially when laboratory inexperience of the researcher can present risks of inaccurate quantities of solutions or incorrect constituents added at any step, or procedure. In considering future adjustments for sample comparison, PCR protocols should again be conditioned to not only suit the species, but the sample type used. As Tiezen *et al*. (1983) highlighted, complexity of soft tissue samples is greater than hair and faeces, and should thus be processed independently.

As the amplified hair samples did not produce sequences that could be used for species investigation, the reasons mentioned above could again be responsible for limitations in molecular outcomes. Further reasons specific to sequencing difficulty may include nucleotide sequence interference during ethanol-based PCR clean up protocols (Nucleics, 2017), as used in this study. Steps should be taken to ensure ethanol is completely absent from the final elution, such as conducting further wash steps from non-ethanol based solutions and ensuring protocols are followed with precision. For this research, amplified samples were quantified, showing concentrations were within the optimal sequencing range, but both samples were measured to be at the lower end of the 20-80 ng/µl, therfore potentially restricting efficiency or length of sequence produced. Another risk is the conditions the samples may be exposed to once the sample is sent away, and the absence of researcher supervision. This can include cleaving of DNA (TSG., n.d.), or failure of primer synthesis (if, for example freeze-thaw events occur).

For future analysis, contamination from ingredient impurities should be avoided as much as possible although as found by Broquet *et al.* (2006) often foreign genetic material poses an unavoidable risk. As suggested by Taberlet *et al.* (1999), detection of contaminates molecules could be applied to indicate, or rule out its potential before adjusting laboratory controls. This study also recommends that further amplifications are made to those samples that have produced DNA bands but fall within the lower end of the optimal range. In this instance, the intention should be to keep DNA concentrations within the optimal range by measuring from the mid-point to higher end of this range in aim of producing a longer and identifiable sequence.

Furthermore, with a wide and varied range of data now available that discusses the effect of natural and applied conditions on non-invasive samples, including techniques and protocols both in and out of the field, and regarding different sample types, it would be useful to collate this information. In other words, with many studies indicating that genetic success is likely to be influenced by each situation, environment, sample type and species; a review of such data would provide both a comprehensive and valuable guide in wildlife research that could be applied to a range of future studies. Such data would consequently be beneficial in promoting non-invasive sampling, taking account of both reliable and accurate scientific research and the ethics of the study subjects.

Despite DNA not producing viable sequences required for species identification, this comparative component of the study revealed hair to be a high quality sample choice that may offer replacement of more intrusively-obtained tissue should processing steps be optimised. Prior to sequence analysis, hair as a sample type exhibited high performance in laboratory procedures, and its use as a positive control in later components of the study demonstrate its potential as a source of useable

DNA in wildlife research. Further study is recommended to determine potential causes of sequencing failure and creating a reliable protocol that provides the greatest opportunity for success.

4.2 Non-Invasive Field Sampling

The implemented bait mechanism was successful in generating bait trap interference where leopard presence was recorded, indicating a mechanism that is effective in both attracting and influencing the actions of the study subjects. A drag method may have further encouraged presence by acting as a bait scent lure, although the true effectiveness and extent of this procedure is not known. Evaluation of past studies such as those of Braczkowski *et al.* (2016) and Patkó *et al*. (2016) were evaluated to recognise that bait scent lures alone cannot be relied upon for comprehensive carnivore study outcomes, but can act as an attractant. Therefore, this study implemented the drag system as an additional supportive procedure only.

The presence of leopards at bait traps occurred only in the Mopaniveld broad habitat classification of Selati Game reserve. This may be because the area encompassed the largest habitat type of the study site and held the majority of the bait traps, but such findings may provide a preliminary indication of preferred habitat type of the leopards in the area. When considered with suggestions of Swanepoel *et al.* (2013) that African leopards will often occupy non-mountainous areas in absence of human conflict, this may indicate why leopards of Selati were only present in the low-lying terrain of Mopani-dominated habitat. A further influence on the distribution and frequency of leopard bait trap presence may be by seasonal climatic conditions (Wilson & Mittermeier, 2009). This study was conducted in the dry season of South Africa when prey abundance is often low due to unfavourable conditions. Since it is a fenced reserve, to reduce the mortality of ungulates by depletion of resources within the closed area, the management of Selati Game Reserve removed several game species two months before this study was conducted. With potential prey choices of the leopard included in the removal, this should be considered a factor that may have heightened response rates of leopards to the baited mechanisms. The distribution of bait traps was set out in attempts to capture different individuals yet one double count of a male at two bait site locations indicted the possible need to re-evaluate the dispersal of bait trap sites. However, with the often extensive home range of male leopards as described by Stein *et al*. (2011), this suggests that double counts in a closed study site may be unavoidable as a male leopard may cover the vast majority of a restricted study site.

Camera trap data, used as part of the complete bait-trap system were effective in identifying individuals from visual analysis of distinguishing features. With the positioning of the camera aimed at capturing the right flank of the leopards, this allowed appropriate comparisons to be made on similar areas of the individuals. Additional characterisation was made by determining the sex of the individuals, and in most cases this was possible due to good quality image data. Where appropriate non-phenotypic features such as a radio tracking collar was used as identification. In the case of individual L1, the camera trap data proved highly advantageous in providing the first sighting of a previously monitored female leopard since failure of the collar in early 2015 (Joubert & Joubert, 2015). Further to this, captured images of a juvenile occurring in the same time period on the same bait trap location, potentially the offspring of L1 provided valuable input in the monitoring of this leopard individual. Without genetic assurance of relatedness, visual data nevertheless provides indication that Selati Game Reserve holds a breeding population of leopards, subsequently allowing insight into the health of both population and ecosystem (Wilson & Mittermeier, 2009). For example, recent camera trap research by *Panthera* (2017) conducting the first population estimate of the Indochinese tiger (*Panthera tigris corbetti*) in Thailand's Eastern Forest Complex captured images of four adults and six cubs (to date). This provided evidence of only the second known breeding population of Indochinese tigers known to exist, and the researchers believe the occurrence may largely be due to extensive forest and tiger protection laws of the area in question. Felid breeding populations can often indicate suitable habitat with adequate resources and low levels of disturbance particularly for the large felids, including leopards that encounter substantial global habitat loss (Wilson & Mittermeier, 2009) and persecution (Athreva *et al.,* 2007). The identification of a juvenile leopard in Selati provides baseline evidence for a suitable habitat type that can hold breeding populations, thus re-emphasing the importance of a conservation strategy or exposing a need to maintain habitat conditions that will allow continued population development. Therefore, irrespective of hair sampling, the bait trap system complete with camera trapping, bait position and overall distribution may prove useful in common ecological surveys such as population density and absence/presence research. Du Preez *et al.* (2014) compared camera-trapping methods using baited and un-baited sites to conduct leopard population surveys, and found significantly increased capture rates on baited sites therefore promoting this as a more efficient method. This study concurs with the research by Du Preez *et al* and proposes additional benefit such as the advantage of restricted camera trap data from baited sites targeting one species, compared to studies that rely upon widely distributed and often extensive camera trap data. As such, findings of this study in addition to previous research support the effectiveness of using a baited system in obtaining phenotypic data of leopards which can then be applied to various ecological research.

Camera trap data of this study could be improved by application of PRESENCE® (11.8) software to estimate patch occupancy rates (and related parameters) if a larger sample size was produced, i.e. if visual data collected over a longer period.

Hair samples were collected from all bait sites in which leopard presence was recorded, demonstrating the efficiency of the adhesive tape in providing a precise sampling contact point. As the follicle contains the important source of DNA, the tape was effective in adhering to the distal, and unrequired part of the hair thus leaving the targeted follicle exposed and less susceptible to contamination of compounds from the adhesive material. In some instances, leopard was the only species that made contact with the tape although some trap interference from the species African civet and Honey badger was recorded by camera trap analysis. This occurrence produced limitations when hair samples were removed from leopards and other species on the same tape and as such, collection had to be conducted carefully in order to try to distinguish species from which the sample(s) originated from. The visual data was useful in determining points of contact by different species but given that hair samples comprise small strands undetectable by camera trap data, categorising the smaller and morphologically-similar hairs by potential species was often subjective. This factor was reduced as much as possible by storing samples in different envelopes according to appearance, area of collection and visual data field analysis but the potential for contamination by non-target species is an obvious risk in these situations. For future research, this study recommends applying small spiny (plant) obstructions at the basal end of the pole to deter non-target species. Similarly when two leopards made contact with the same bait trap, distinguishing between individuals from those samples in the field proved difficult. In addition, the bait may have been a source of either sample contamination or disturbance that could affect later laboratory outcomes. To restrict this, the pelt was removed to limit cross-hair contamination and wire was used to secure that bait, yet there remained the risk of the bone (by which the wire was placed around) breaking and making contact with the tape. In this study the wire secured the bait with a double layer of flexible wire but in future efforts, it would be advisable to introduce a standardised and stronger technique in bait placement (e.g. double wire layer wrapped four times around the vertebral column).

This study was advantageous in combining food appetence, natural (climbing) behaviour and selective sampling thus allowing consistent and adequate sample collection and indicating the potential of the field methodology in hair sampling. In addition, camera trap evidence demonstrated an effective monitoring technique in its own right. Future studies should be conducted to examine the effect of the drag procedure, possibly by recording tracks and signs, or implementing camera

trap transects to evaluate the effectiveness of using bait drag (scent) lures to influence leopard movement. Furthermore, the study should be repeated in parallel dry season conditions, when prey abundance is likely to be at a similarly reduced level, either through natural depletion or following management strategies such as those implemented before this study. Repetition of the study in Selati and across comparable regions would also allow a clearer understanding of associations between habitat and leopard distribution that may indicate habitat preferences within Selati Game Reserve, and throughout South Africa. In addition camera traps were limited to one per site thus restricting the capacity of visual data to one flank and one specified area, possibly missing leopard presence close to, but not within the camera range of the bait mechanism. An alternative to this would be to conduct direct observations over a long period, but such a procedure is time consuming, poses risks to the observer (in presence of dangerous game) (Athreva *et al.,* 2010) and may deter leopards entering the area (Swanepoel *et al.,* 2013). Therefore, as found by Du Preez *et al.* (2014), the implementation of more extensive camera trap distribution as supplement to baited sites would likely prove more efficient in leopard detection. Allocating a second camera in each site would allow capture of individuals from both flanks, possibly reducing the frequency of unidentifiable individuals upon analysis. Added placements of camera traps in the area surrounding the bait site could also identify whether some leopards are enticed by the bait lure but deterred by other factors (e.g. human-scent on the bait mechanism). Furthermore, this strengthened camera trap support would allow clearer determination of species and individuals that had made contact with bait trap. The success encountered with this study and those similar adhesion-based techniques applied by Sloane *et al.* (2000) and Walker *et al.* (2006) can warrant future investigation into the efficiency of the field methodology and its applicability to leopards and other mammalian species in non-invasive genetics.

4.3 Genetic Efficiency of Non-Invasive Sampling

Samples that were recorded as having the highest concentrations of DNA resulted in successful amplification, whereas those with concentrations of less than 1 ng/µl did not amplify. This suggests that a minimum quantity of DNA is required in order for amplification. As Taberlet *et al.* (1999) described, a major limitation of non-invasive samples is the low yield of host DNA, a particular issue with the small hair follicle sample choice. It is possible that the samples that yielded the lowest DNA required multiple PCR applications. A minimum of 20 ng/µl DNA is required for visibility of the agarose gels, and so once an optimal PCR procedure is identified for the sample types (possibly by use of positive control), it may be advisable to attempt multiple PCR applications, or simply increase the number of extension cycles. The samples with the purest primary and secondary ratios were

successfully amplified, and thus these purity values may be a first indication of samples that are likely to amplify through PCR. It should be recognised however that limitations in sample size do not allow sufficient analysis of the reliability of these readings in predicting PCR outcomes.

Ten amplifications were made from fifteen samples, demonstrating adequate storage, number of hairs used and extraction procedures. While the small sample size restricts a thorough examination of optimal sample processes, past literature supports the sample processing of this study. For example, reviews by Taberlet *et al.* (1999) and U.S Fish & Wildlife Service (2017) support the desiccation of hair as a standardised preservation condition in most environments. An additional storage by ethanol condition was adopted for this study but did not show any obvious differences in laboratory performance, thus offering an additional hair preservation option. The lowest numbers of hair where amplification occurred was four, supporting the sample type as a viable source of DNA even when only a small sample product is available. Extraction was conducted by two methods in an attempt to obtain higher DNA yields, but amplifications were made following both procedures and so this study does not support one extraction method over another. The visible amplifications were made following PCR protocols by Forgham *et al*. (2015) by use of the *cytb* forward and reverse primers, supporting the effectiveness of primer choice and conditions from this previous study. Conversely, PCR conditions following carnivore-specific protocols set forth by *Carnivora* (2011) using COI primers, Chaves *et al*. (2011) using *ATP6* primers, and a further PCR application by Branicki *et al*. (2016) using the same *cytb* primers as Forgham *et al.*, (2015) did not produce visible DNA bands. This was unusual with regards to the carnivore-specific primers by which the same protocols had previously been used with success in felid research. As such, the outcomes of this study indicate potential problems with the PCR process which may be through human error, contamination, inadequate PCR conditions (regarding sample qualities or suitability to primers), or primer choice being inadequately matched to sample type or species. Possible reasons where human error could have been responsible for negative PCR outcomes includes mistakes in procedure and constituents. As indicated by Broquet *et al.* (2006), a common limitation of non-invasive population genetics is sample susceptibility to contamination from foreign genetic material. Further contamination that may interfere with the PCR process can included ingredient impurities, especially when conducting numerous amplification trials using the same stock supplies. The issue of primer suitability was a particularly prominent complication of PCR methodology described by Broquet *et al*. (2006) and Wilcox *et al.* (2013) when potentially already degraded samples are also susceptible to species-bias through primer choice.

In sequencing, the target species was not detected, but two matches were identified from the database. In examining the DNA concentrations and purity values of these samples, it was clear that LPH01 produced the highest concentrations within the optimal sequencing range, although neither produced the best purity values. This suggests that further analysis of DNA concentrations could be made to benefit future research regarding optimal DNA concentrations, where a larger number of sequenced models are available for examination. However, with regards to the purity of samples, this quality indictor does not appear to be a contributing factor in the non-invasive hair sampling of leopards but again; further analysis is recommended where a larger sample size is available. Considering the results of this study alongside the findings of Wilcox *et al.* (2013) which also described the risk of primer bias when detecting a number of possible species, this indicates that primer choice is a possible factor in determining why non-target species were matched. The DNA sequence of the leopard may not have been optimally compatible with those primers chosen. As a universal primer, *cytb* is advantageous in its sensitivity to a wide range of sympatric species (Branicki *et al.,* 2003) but such suitability poses a risk of bias towards particular species, or taxonomic groups over others.

The first sequence alignment match was the African Civet, of which produced the highest sequence alignment match of 98%. This result directly indicates the complications that can occur by contamination from individuals other than the target species. Nonetheless, this outcome also demonstrates the effectiveness of the field methodology and its potential in obtaining genetic data of various species that can make contact with the bait trap. This sample was used as a positive control in a second sequencing trial and gave the same result to again support the quality of the sample and it's usability as a repeatable component. The second sequence produced a 92% sequence alignment match with bovine, suggesting contamination with the bait used in the field mechanism. As the skin and pelt of the bait was removed to minimise this risk, and actions were taken to target leopard hair over other species that had made contact with bait site; such matches of this study agree with those factors reported by Wilcox *et al.* (2013) suggesting that the specificity of the primers used in PCR may be biased towards the African civet and the bovine species. As such, a primer check was made using the same sequence database. The sequences available for both African civet and bovine species produced a lower number of mismatches in alignment with the primer sequences than when checking against available leopard sequences. Such results indicate that the universal *cytb* primers are actually better suited to those species that were matched, and therefore potentially limiting the sequencing outcomes of this study. Bhagavatula & Singh (2006) designed tiger-specific primers to distinguish accurate faeces-origin, and Mongol *et al.* (2009) designed leopard specific primers in microsatellites-based analysis. In review, more specific primers such as

felid, or the design of leopard-specific primers for this research may have eliminated the species-bias problems.

The other major risk factor associated with non-invasively sampling, and previously mentioned as a potential factor of non-sequencing for the lion biological sample comparisons, is occurrence of genotyping errors. As reported by Goossens *et al*. (1998), Taberlet *et al.* (1999) and Piggott & Taylor (2003) the common non-invasive sample types (hair and faeces) and more importantly, the DNA within is considered highly susceptible to degradation. The potential effect of this factor is consistently discussed across many non-invasive genetic studies (Wasser *et al*., 1997; Farrell *et al*., 2000; Piggot & Taylor, 2003 and Penasco *et al*., 2011). This degradation can not only affect quality of DNA but produce problems by sample deterioration leading to flawed genotypes, an event which occurs primarily due to false alleles and allelic dropout. Often these errors cannot be avoided due to limitations in current knowledge regarding the methods of determining error rates, and hence should be quantified to allow interpretation and the extent of potential error in findings (Broquet & Petit, 2004).

In considering future research, a larger sample size would improve this study by allowing exploration of the factors that may limit or alter laboratory outcomes. For instance, the effect of DNA purity and concentrations (for sequencing analysis) could be examined with more assurance in understanding the influence of these factors. Furthermore, as mentioned with the lion sample comparison, comprehensive analysis into the effects of various storage, extraction and PCR procedures on leopard hair samples in particular would ensure optimal conditions are available for future research.

With primer availability limited to carnivore-species sequences for this study, further research would also benefit from primers designed specifically to detect the leopard. Time restrictions were a limiting factor of this study but PCR procedures including programme conditions (such as annealing temperatures and number of cycles), in addition to primers should be trialled to a greater extent, thus allowing more precise and appropriate alterations to be made encompassing those numerous potential reasons for non-amplification. For instance, if the leopard-appropriate *COI* and *ATP6* primers (*Carnivora*, 2011; Chaves *et al*., 2011) and conditions were adjusted to produce successful amplifications, sequencing of the leopard DNA may have resulted. With regards to susceptibility of DNA to degradation and implication of genotyping errors, the measurements of those incidents should be addressed for suitable interpretation in findings. The detection of genotyping errors traditionally relies on the comparison of the non-invasive incident rates with the rates from reference sequences obtained from other sources, such as tissue (Wilcox *et al.,* 2013). An integration of this technique as a development on the first component of this study could strengthen its findings

and conclusions on the sample comparison component of this study. Further benefits could be achieved if downstream genetic applications applied not just to distinguish species, but specific individuals (e.g. by use of microsatellite genetic markers). Through non-invasive sampling, Mondol *et al*. (2009) applied felid-specific microsatellites to estimate the minimum number of leopards in a human-leopard conflict area in Western India. Results held important implications for conflict management and conservation of the leopard population in the area of concern. Similar technique in this study could consequently eliminate the field limitations whereby more than one leopard makes contact, and provide much more detailed information about the population being studied.

Chapter 5. Summary

In order to investigate the viability of non-invasive genetics, this study focused on three aims in order to compare sample types, implement a new field technique and test the efficiency of the field methodology in obtaining genetic information. The first component tested two common non-invasive sample types (faeces and hair) with a traditionally-used invasive substitute (tissue). The second investigated the underlying field procedures required to successfully obtain hair samples non-invasively, and the third sought to bring the first two aims together and explore the effectiveness of the field method in obtaining molecular information of the leopard.

In comparing sample types of lion, hair proved the best in quantity and quality for this study. As the only sample type that was amplified, the usability of this DNA extracted from the hair was demonstrated by its application as a positive control for later PCR trials of DNA sourced from leopard hair. Tissue was the next greatest in quantity and quality following DNA extraction yet no amplifications were made by this, or the faecal samples; in which the latter produced the lowest quantity and quality values. Importantly, this study does highlight the extensive literature that categorises tissue as a more efficient and useable source of DNA than non-invasive alternatives despite its non-amplification for this study. Nevertheless, findings of this study do certainly signify the potential for hair, not only as a viable non-invasive source of DNA, but one that may also hold the potential to replace invasive alternatives, if suitable field methodology and optimal laboratory procedures are applied.

These initial findings supported the next component of the study in which an innovative baited hair-trap system was implemented in order to encourage targeted leopard interference. With inconsistencies in field techniques and success rates evidenced in past literature, this study proposed a procedure never before applied to felids. The field methodology demonstrated high efficiency by obtaining hair samples in all bait traps were leopards were recorded as present. The complete field system, including bait trap mechanism and accompanying camera trap data produced an effective method that could be applied as a useful ecological surveying and monitoring tool for leopards with further implications for habitat and species conservation management.

To link and validate the field technique to genetic analysis, laboratory procedures were conducted in aim of gaining genetic information from the leopard hair samples. Amplification only occurred by use of *cytb* universal primers, and sequencing was not successful in identifying leopard as the origin of the DNA. Two important factors of this included genotyping errors, consistent across non-invasive sampling in previous literature (Gerloff *et al*., 1995; Goossens*, et al*., 1998; Taberlet *et al*., 1999 and

Piggott & Taylor, 2003), and specificity of primers biased towards species other than the leopard. Successful sequencing did occur by African civet and detection of bait-originated contamination also resulted. Implications of this component of the study included the potential of obtaining useable DNA from leopards if the most suitable primers were applied, with further applicability of the method to other species and downstream applications in possible future research.

The integration of the three components of this study produced a comprehensive analysis that can be used for future studies of non-invasive sampling. This study discusses the potential of hair as a high quality source of DNA and provides a baseline technique that warrants further investigation into non-invasive hair sampling of leopards. The results presented here demonstrate how non-invasive sampling can be employed to provide genetic information on elusive species, but highlights how processing steps should be optimised for the specific study subject. Important factors to consider include storage to reduce degradation for PCR applications and an emphasis on primer suitability to improve molecular results and benefit future research. This study discusses how ethical considerations highlight the importance of non-invasive sampling, exposes the lack of consistent sample processing techniques, recognises factors that currently limit the viability of non-invasive genetics and presents an effective method for non-invasive genetic sampling of elusive species.

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Appendices

Appendix I. Extraction method one.

Wizard® SV Genomic DNA Purification System

INSTRUCTIONS FOR USE OF PRODUCTS A2360, A2361 AND A2365.

Quick PROTOCOL

Purification of Genomic DNA from Mouse Tail Clippings or Animal Tissues Using a Microcentrifuge

Sample Preparation

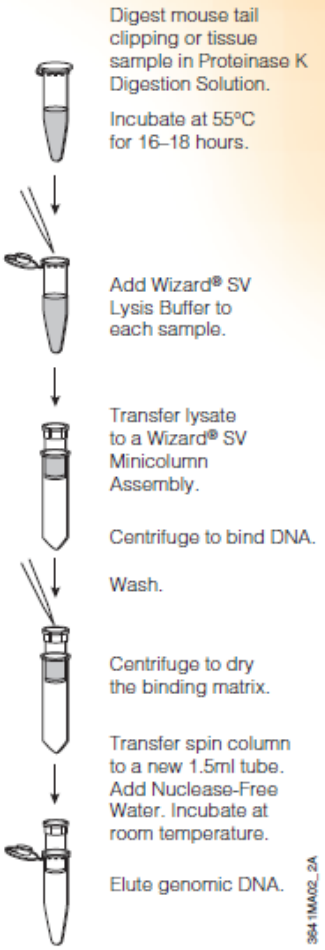
1. Cut a 0.5 to 1.2cm length of mouse tail or weigh up to 20mg of tissue sample. Cut the clipping or tissue sample into two pieces and place them in a 1.5ml microcentrifuge tube.
2. Add 275µl of Digestion Solution Master Mix to each tube.

Digestion Solution Master Mix	Volume per Sample
Nuclei Lysis Solution	200µl
0.5M EDTA (pH 8.0)	50µl
Proteinase K, 20mg/ml	20µl
RNase A Solution, 4mg/ml	5µl
Total Volume	**275µl**

3. Incubate the sample tubes overnight (16–18 hours) in a 55°C heat block.
4. Add 250µl of Wizard® SV Lysis Buffer to each sample. Vortex.
5. Process the lysate as soon as possible after adding the Lysis Buffer. If frozen at –70°C, lysates should be thawed and heated at 55°C for one hour prior to processing. Lysates must be warm for processing.

Purification of Genomic DNA from Lysate Using a Microcentrifuge

6. Transfer each sample lysate from the 1.5ml tube to a separate Wizard® SV Minicolumn Assembly.
7. Spin the Assembly at 13,000 × *g* for 3 minutes.
8. Remove minicolumn from the Assembly and discard the liquid in the Collection Tube. Replace the minicolumn into the Collection Tube.
9. Add 650µl of Column Wash Solution (CWA; with 95% ethanol added) to each assembly. Centrifuge at 13,000 × *g* for 1 minute. Discard the liquid from the Collection Tube. Repeat this step for a total of 4 washes.
10. Discard the liquid from the Collection Tube and reassemble the minicolumn assembly. Centrifuge for 2 minutes at 13,000 × *g* to dry the binding matrix.
11. Transfer the Wizard® SV Minicolumn to a new 1.5ml tube. Add 250µl of room temperature Nuclease-Free Water. Incubate for 2 minutes at room temperature.
12. Centrifuge the minicolumn/elution tube assembly at 13,000 × *g* for 1 minute. Do not discard the liquid in the elution tube.
13. Add an additional 250µl of Nuclease-Free Water and incubate at room temperature for 2 minutes. Centrifuge the minicolumn/elution tube assembly at 13,000 × *g* for 2 minutes.
14. Remove the minicolumn and store the purified DNA at –20 to –70°C.



See additional protocol information in Technical Bulletin #TB302, available online at:
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Appendix II. Extraction method two.

Protocol: Isolation of DNA from Stool for Human DNA Analysis

Lysis conditions in this protocol are optimized to increase the ratio of human DNA to nonhuman DNA. Nonhuman DNA is not excluded by this procedure.

Important points before starting

- All centrifugation steps should be carried out at room temperature (15–25°C) at 20,000 x *g* (approximately 14,000 rpm). Increase the centrifugation time proportionately if your centrifuge cannot provide 20,000 x *g* (e.g., instead of centrifuging for 5 min at 20,000 x *g*, centrifuge for 10 min at 10,000 x *g*).
- The 2 ml tubes used in step 4 should be wide enough to accommodate an InhibitEX Tablet.

Things to do before starting

- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on the labels.
- Mix all buffers before use.
- If a precipitate has formed in Buffer ASL or Buffer AL, dissolve by incubating at 70°C.
- Prepare a 70°C water bath for use in step 11.

Procedure

1. **Weigh 180–220 mg stool in a 2 ml microcentrifuge tube (not provided) and place tube on ice.**

   This protocol is optimized for use with 180–220 mg stool but can also be used with smaller amounts. There is no need to reduce the amounts of buffers or InhibitEX matrix when using smaller amounts of stool. For samples >220 mg, see "Protocol: Isolation of DNA from Larger Volumes of Stool", page 30.

   If the sample is liquid, pipet 200 µl into the microcentrifuge tube. Cut the end of the pipet tip to make pipetting easier.

   If the sample is frozen, use a scalpel or spatula to scrape bits of stool into a 2 ml microcentrifuge tube on ice.

   Note: When using frozen stool samples, take care that the samples do not thaw until Buffer ASL is added in step 2 to lyse the sample; otherwise the DNA in the sample may degrade. After addition of Buffer ASL, all following steps can be performed at room temperature (15–25°C).

2. **Add 1.6 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.**

   Note: It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final eluate.

3. **Centrifuge sample at full speed for 1 min to pellet stool particles.**
4. **Pipet 1.4 ml of the supernatant into a new 2 ml microcentrifuge tube (not provided) and discard the pellet.**

   Note: The 2 ml tubes used should be wide enough to accommodate an InhibitEX Tablet.

   Transferring small quantities of pelleted material will not affect the procedure.

5. **Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.**
6. **Centrifuge sample at full speed for 3 min to pellet stool particles and inhibitors bound to InhibitEX matrix.**

   Note: For most samples, 3 min centrifugation is sufficient. With some samples, however, centrifugation for 3 min may result in a pellet that is not sufficiently compact. Therefore it may be difficult to remove enough supernatant to transfer 600 µl supernatant after the next centrifugation step (step 9). In these cases, we recommend to centrifuge for 6 min.

   Note: When processing more than 12 samples, for this step and step 7 we recommend processing batches of no more than 12 samples each. This is because the pellets formed after centrifugation will break up quickly if the supernatant is not removed immediately.

7. **Immediately after the centrifuge stops, pipet all of the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min.**

   Transferring small quantities of pelleted material from step 6 will not affect the procedure.

8. **Pipet 25 µl proteinase K into a new 2 ml microcentrifuge tube (not provided).**
9. **Pipet 600 µl supernatant from step 7 to the 2 ml microcentrifuge tube containing proteinase K.**
10. **Add 600 µl Buffer AL and vortex for 15 s.**

    Note: Do not add proteinase K directly to Buffer AL.

    It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.

11. **Incubate at 70°C for 10 min.**

    Centrifuge briefly to remove drops from the inside of the tube lid (optional).

12. **Add 600 µl of ethanol (96–100%) to the lysate, and mix by vortexing.**

    Centrifuge briefly to remove drops from the inside of the tube lid (optional).

13. **Label the lid of a new QIAamp spin column provided in a 2 ml collection tube. Carefully apply 600 µl lysate from step 12 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.**

    Close each spin column in order to avoid aerosol formation during centrifugation.

    If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

14. **Carefully open the QIAamp spin column, apply a second aliquot of 600 µl lysate and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.**

    Close each spin column in order to avoid aerosol formation during centrifugation.

    If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

15. **Repeat step 14 to load the third aliquot of the lysate onto the spin column.**

16. **Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.**

17. **Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.**

    Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

18. **Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.

19. **Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided). Carefully open the QIAamp spin column and pipet 200 µl Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA.**

Note: When using eluates in PCR, for maximum PCR robustness we highly recommend adding BSA to a final concentration of 0.1 µg/µl to the PCR mixture. For maximum PCR specificity we recommend using QIAGEN HotStarTaq *Plus* DNA Polymerase (see ordering information on page 39). For best results in downstream PCR, use the minimum amount of eluate possible in PCR; the volume of eluate used as template should not exceed 10% of the final volume of the PCR mixture. Also, note that high amounts of template DNA may inhibit the PCR.

DNA yield is typically 15–60 µg but, depending on the individual stool sample and the way it was stored, may range from 5 to 100 µg. DNA concentration is typically 75–300 ng/µl.

For more information about elution and how to determine DNA yield, purity, and length, see the Appendix, page 36.

For long-term storage, we recommend keeping the eluate at –20°C.

Appendix III. Extraction method three

Protocol: Isolation of Total DNA from Nail Clippings and Hair

This protocol is for isolation of total (genomic and mitochondrial) DNA from nail clippings and hair roots or shafts.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Check whether carrier RNA is required (see pages 10 and 12).

Things to do before starting

- Equilibrate Buffer ATE or distilled water for elution to room temperature (15–25°C).
- Set a thermomixer or heated orbital incubator to 56°C for use in step 2 and (optional) step 15, and a second thermomixer or heated orbital incubator to 70°C for use in step 5. If thermomixers or heated orbital incubators are not available, heating blocks or water baths can be used instead.
- If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation.
- Prepare an aqueous 1 M DTT (dithiothreitol) stock solution. Store aliquots at –20°C. Thaw immediately before use.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 11.

Procedure

1. Lyse the samples according to step 1a for nail clippings, step 1b for hair roots, or step 1c for hair shafts (without roots).

1a. Transfer the nail clippings to a 1.5 ml microcentrifuge tube (not provided). Add 300 µl Buffer ATL, 20 µl proteinase K, and 20 µl 1 M DTT. Close the lid and mix by pulse-vortexing for 10 s. Continue the procedure from step 2.

1b. Add 300 µl Buffer ATL, 20 µl proteinase K, and 20 µl 1 M DTT to a 1.5 ml microcentrifuge tube (not provided). Cut off a 0.5–1 cm piece starting from the hair bulb and transfer it to the 1.5 ml microcentrifuge tube. Close the lid and mix by pulse-vortexing for 10 s. Continue the procedure from step 2.

1c. Add 300 µl Buffer ATL, 20 µl proteinase K, and 20 µl 1 M DTT to a 1.5 ml microcentrifuge tube (not provided). Cut the hair into 0.5–1 cm pieces, and transfer them to the 1.5 ml microcentrifuge tube. Close the lid and mix by pulse-vortexing for 10 s. Continue the procedure from step 2.

2. **Place the tube in a thermomixer or heated orbital incubator, and incubate at 56°C with shaking at 900 rpm for at least 1 h.**

   In general, hairs are lysed in 1 h. If necessary, increase the incubation time to ensure complete lysis.

   If using a heating block or water bath, vortex the tube for 10 s every 10 min to improve lysis.

   For larger samples of nail clippings, we recommend overnight incubation at 56°C. Any material that is not lysed during this incubation step or the incubation in step 5 will be pelleted during centrifugation in step 6.

3. **Briefly centrifuge the tube to remove drops from the inside of the lid.**

4. **Add 300 µl Buffer AL, close the lid, and mix by pulse-vortexing for 10 s.**

   To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution.

   A white precipitate may form when Buffer AL is added to Buffer ATL. The precipitate does not interfere with the QIAamp procedure and will dissolve during incubation in step 5.

   Note: If carrier RNA is required (see page 10), add 1 µg dissolved carrier RNA to 300 µl Buffer AL. Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer ATE and then added to Buffer AL.

5. **Place the tube in the thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900 rpm for 10 min.**

   If using a heating block or water bath, vortex the tube for 10 s every 3 min to improve lysis.

6. **Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.**

7. **Add 150 µl ethanol (96–100%), close the lid, and mix thoroughly by pulse-vortexing for 15 s.**

   To ensure efficient binding in step 9, it is essential that the sample and ethanol are thoroughly mixed to yield a homogeneous solution.

8. **Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.**

9. **Carefully transfer the supernatant from step 8 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim.**

10. **Close the lid, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

    If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty.

11. **Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

12. **Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

    Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column.

13. **Carefully open the QIAamp MinElute column and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.**

14. **Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min to dry the membrane completely.**

    This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications.

15. **Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate at room temperature (15–25°C) for 10 min or at 56°C for 3 min.**

16. **Apply 20–50 µl Buffer ATE or distilled water to the center of the membrane.**

    Important: Ensure that Buffer ATE or distilled water is equilibrated to room temperature. Dispense Buffer ATE or distilled water onto the center of the membrane to ensure complete elution of bound DNA.

    QIAamp MinElute columns provide flexibility in the choice of elution volume. Choose a volume according to the requirements of the downstream application. Elution with small volumes increases the final DNA concentration in the eluate significantly, but reduces the overall DNA yield. Remember that the volume of eluate will be up to 5 µl less than the volume of elution solution applied to the column.

17. **Close the lid and incubate at room temperature for 1 min. Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 1 min.**

    Incubating the QIAamp MinElute column loaded with Buffer ATE or water for 5 min at room temperature before centrifugation generally increases DNA yield.

Appendix IV. Extraction method four

Step 1:

Hairs were examined to identify those with a follicle. The number to be used in the extraction was recorded

Step 2:

Hair roots were cut to size as per previous extraction method and each sample placed in a sterile 1.5 ml Eppendorf. This was labelled with the sample number, researcher initials and date.

500µl Buffer 1 and 20 µl Proteinase K (20 mg/ml) was then added to each Eppendorf (Promega, 2012).

The samples were each vortexed for 10 seconds and checked to ensure all samples material were immersed in the solution. The samples were incubated overnight (16 hours) to allow digestion.

Step 3.

The samples were removed from the heatblock and 500 µl chloroform: isoamyl alcohol (24:1) was added to each. Mixing occurred by inverting each sample for 10 seconds.

The samples were centrifuged for ten minutes at 14 000 rpm. After this time, three layers were visible. The top layer in which contained the nucleic acid was pipetted into a new sterile 1.5 ml Eppendorf. Pipetting occurred gradually whilst recording the final transferred quantity. This was carried out for each sample.

An equal amount of chloroform: isomyl alcohol (24:1) was added to each sample and mixed by 10 seconds of inverting.

The samples were centrifuged for ten minutes at 14 000 rpm and top layer again pipetted, with quantity recorded into a new Eppendorf for each sample.

The DNA was precipitated by the addition of 0.08 volumes of cold 3 M sodium acetate and an equal quantity of 75% cold ethanol into each. The samples were then placed in the freezer for one hour (minimum).

The samples were centrifuged for ten minutes at 14 000 rpm which allowed the formation of a pellet. The liquid above the samples was pipetted out taking care to ensure the clear pellet remained at the base of the Eppendorf.

To wash: 300 µl cold 75% ethanol was added to each pellet before being centrifuged for two minutes at 14 000 rpm.

The liquid was again carefully pipetted out taking care not to remove the clear pellet. 300 µl cold 75% ethanol was again added to each sample and centrifuged for five minutes at 14 000 rpm.

Liquid was carefully removed and the pellet dried by centrifuging (without liquid) for five minutes at 14 000 rpm.

The pellet was further dried by incubating at room temperature (no lid) for ten minutes, or until no traces of ethanol could be seen.

The DNA was re-suspended by adding 50 µl Elution buffer (AE) buffer and incubated (with lid on) at 55°C.

The samples were stored at -20°C until required.

Appendix V. Extraction method four stock solution constituents.

1 M Tris pH 8, 100 ml

Tris (Fisher Scientific)	12.11 g
Distilled water (dH_2O)	70 ml

Dissolve Tris and bring to 90 ml

pH to 8 with concentrated Hydrochloric acid (HCl) (Sigma), approximately adding 5 ml progressively

Bring to 100 ml

0.5 M Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) pH 8, 100 ml

EDTA (Fisher Scientific)	18.612 g
dH_2O	75 ml
Sodium Hydroxide pellets (NaOH) (Sigma)	2 g

Slowly add more NaOH while mixing until reaches pH 8

5 M NaCL 100 ml

Sodium Chloride (NaCL) (Fisher Scientific)	29.22 g
dH_2O	70 ml

Dissolve and bring to 100 ml

Note. The first 3 stocks need to be autoclaved.

The above stocks are required for CTAB Buffer

CTAB Buffer

1 M Tris	10 ml
5 M NaCl	28 ml
0.5 M EDTA	4 ml
Hexadecyltrimethylammonium bromide (CTAB) (Acros Organics)	2 g

Overnight incubation period may be needed to aid dissolving.

AE Buffer (Qiagen®)

10 mM Tris-Cl

0.5 mM EDTA; pH 9.0.

Buffer 1

CTAB Buffer	2 ml
Polyvinylpyrrolidone (PVP) (Sigma Aldrich)	0.08 g
Dithiothreitol (DTT) (Sigma Aldrich)	10 µl
dH_2O to 100 ml (add small amounts at a time)	

EDTA (1 ltr, 0.5 M, pH 8.0)

EDTA (for 200 ml 37.22 g)	186.1 g
dH_2O	800 ml
Stir vigorously on magnetic stirrer	
pH to 8.0 with NaOH (approx. 20 g) add slowly	

Appendix VI. Nanodrop 2000 Purity Ratios

Thermo SCIENTIFIC

T009-TECHNICAL BULLETIN NanoDrop 1000 & 8000

260/280 and 260/230 Ratios

Absorbance measurements made on any spectrophotometer, including the Thermo Scientific NanoDrop™ 1000 Spectrophotometer and the NanoDrop™ 8000 Spectrophotometer, will include the absorbance of all molecules in the sample that absorb at the wavelength of interest. Since nucleotides, RNA, ssDNA, and dsDNA all absorb at 260 nm, they will contribute to the total absorbance of the sample. Therefore, to ensure accurate results, nucleic acid samples will require purification prior to measurement.

260/280

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Some researchers encounter a consistent 260/280 ratio change when switching from a standard cuvette spectrophotometer to the NanoDrop1000 Spectrophotometer. The three main explanations for this observation are listed below:

CHANGE IN SAMPLE ACIDITY

Small changes in the pH of the solution will cause the 260/280 to vary*. Acidic solutions will under-represent the 260/280 ratio by 0.2-0.3, while a basic solution will over-represent the ratio by 0.2-0.3. If comparing results obtained using the either NanoDrop 1000 or the NanoDrop 8000 to results obtained using other spectrophotometers, it is important to ensure that the pH of an undiluted sample measured on our instruments is at the same pH and ionic strength as the diluted sample measured on the conventional spectrophotometer.

* William W. Wilfinger, Karol Mackey, and Piotr Chomczynski, Effect of pH and Ionic Strength on the Spectrophotometric Assessment of Nucleic Acid Purity: *BioTechniques 22:474-481 (March 1997)*

WAVELENGTH ACCURACY OF THE SPECTROPHOTOMETERS

Although the absorbance of a nucleic acid at 260 nm is generally on a plateau, the absorbance curve at 280 nm is quite steeply sloped. A slight shift in wavelength accuracy will have a large effect on 260/280 ratios.

For example, a +/- 1 nm shift in wavelength accuracy will result in a +/- 0.2 change in the 260/280 ratio. Since many spectrophotometers claim a 1 nm accuracy specification, it is possible to see as much as a 0.4 difference in the 260/280 ratio when measuring the same nucleic acid sample on two spectrophotometers that are both within wavelength accuracy specification.

NUCLEOTIDE MIX IN YOUR SAMPLE

The five nucleotides that comprise DNA and RNA exhibit widely varying 260/280 ratios**. The following represent the 260/280 ratios estimated for each nucleotide if measured independently:

Guanine:	1.15
Adenine:	4.50
Cytosine:	1.51
Uracil:	4.00
Thymine:	1.47

The resultant 260:280 ratio for the nucleic acid being studied will be approximately equal to the weighted average of the 260/280 ratios for the four nucleotides present. It is important to note that the generally accepted ratios of 1.8 and 2.0 for DNA and RNA respectively, are "rules of thumb". The actual ratio will depend on the composition of the nucleic acid. Note: RNA will typically have a higher 260/280 ratio due to the higher ratio of Uracil compared to that of Thymine.

** Leninger, A. L. Biochemistry, 2nd ed., Worth Publishers, New York, 1975

Appendix VII. Non-Amplification PCR Trials.

1.

C*ytb* primers (126 bp length):

Forward primer: 5'-CCATCCAACATCTCCGCATGATGAAA-3'
Reverse primer: 5'-CCCCTCAGAATGATATTTGGCCTCA-3'

PCR master mix per sample:

Taq buffer	0.5 µl
Deoxynucleotides (DNTPs)	0.5 µl
Taq polymerase	1 µl
Magnesium Chloride ($MgCl_2$)	1.5 µl
Forward primer (diluted 1:10 original stock solution: Nuclease-free water)	1 µl
Reverse primer (diluted 1:10 original stock solution: Nuclease-free water)	1 µl

Between 1 – 5 µl of extracted DNA template is also added based upon ng/µl Nanodrop reading per sample. Up to but no greater than 250 ng/µl is added to each sample. Dilutions were adjusted in further PCR trials (1:10 and 1:5).

Each sample of master mix and DNA template is pipetted into sterile PCR tubes and each filled to a total volume of 25 µl. A negative control with no DNA template was added with each PCR operation.

PCR programming (Chaves *et al*., 2011):

Initial denaturation: 94°C/5’
Denaturation: 94°C/1’
Annealing: 50°C/30”
Extension: 72°C/1’ (30 cycles of denaturation, annealing, elongation)
Final extension: 72°C/10’
Storage: 4°C on infinite setting (until removal).

2.

ATP6 primers (126 bp length):

Forward primer sequence: 5'-AACGAAAATCTATTCGCCTCT-3'

Reverse primer sequence: 5'-CCAGTATTTGTTTTGATGTTAGTTG-3'

PCR master mix per sample:

Taq buffer	2.5µl
Deoxynucleotides (DNTPs)	0.5µl
Taq polymerase	1µl
Forward primer (diluted 1:10 original stock solution: Nuclease-free water)	0.5µl
Reverse primer (diluted 1:10 original stock solution: Nuclease-free water)	0.5µl

Between 1 – 5 µl of extracted DNA template is also added based upon ng/µl Nanodrop reading per sample. Up to but no greater than 250 ng/µl is added to each sample. Dilutions were adjusted in further PCR trials (1:10 and 1:5).

Each sample of master mix, DNA template is pipetted into sterile PCR tubes and each filled to a total volume of 25 µl. A negative control with no DNA template was added with each PCR operation.

PCR programming (*Carnivora*, 2011):

Initial denaturation: 94°C/3’

Denaturation: 94°C/45”

Gradient 30 cycles: 94°C/45”, 50°C/45”, 72°C 1’30” (annealing temperature reduced from 60°C by 2°C until final annealing temperatures of 50°C after 10; 5; 5; 5; 5; 10 cycles)

Final extension: 72°C/3’

Storage: 4°C on infinite setting (until removal).

3. *ATP6* primers (126 bp length):

Forward primer sequence: 5'-AACGAAAATCTATTCGCCTCT-3'

Reverse primer sequence: 5'-CCAGTATTTGTTTTGATGTTAGTTG-3'

PCR master mix per sample:

Taq buffer	2.5 µl
Deoxynucleotides (DNTPs)	0.5 µl
Taq polymerase	1 µl
Forward primer (diluted 1:10 original stock solution: Nuclease-free water)	1 µl
Reverse primer (diluted 1:10 original stock solution: Nuclease-free water)	1 µl

Between 1 – 5 µl of extracted DNA template is also added based upon ng/µl Nanodrop reading per sample. Up to but not greater than 250 ng/µl is added to each sample. Dilutions were adjusted in further PCR trials (1:10 and 1:5).

Each sample of master mix, DNA template is pipetted into sterile PCR tubes and each filled to a total volume of 25 µl. A negative control with no DNA template was added with each PCR operation.

PCR programming: (Haag *et al*., 2009)

Denaturation: 94°C/45”
Touchdown 10 cycles: 94°C/45”, 50°C/45”, 72°C 1’30” (annealing temperature reduced from 60°C by 1°C until final annealing temperature of 50°C)
94°C/45”, 50°C/45”, 72°C 1’30” (30 cycles)
Final extension: 72°C/3’
Storage: 4°C on infinite setting (until removal).

Appendix VIII. PCR clean-up protocol.

Wizard® SV Gel and PCR Clean-Up System

INSTRUCTIONS FOR USE OF PRODUCTS A9280, A9281, A9282, AND A9285.

Quick PROTOCOL

DNA Purification by Centrifugation

Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65°C until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.
2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.
3. Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × *g* for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.
5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × *g* for 5 minutes.
6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.
8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × *g* for 1 minute.
9. Discard Minicolumn and store DNA at 4°C or –20°C.

3TG061A07_2A

Additional protocol information is available in Technical Bulletin #TB308, available online at:
www.promega.com

ORDERING/TECHNICAL INFORMATION:
www.promega.com • Phone 608-274-4330 or 800-356-9526 • Fax 608-277-2601

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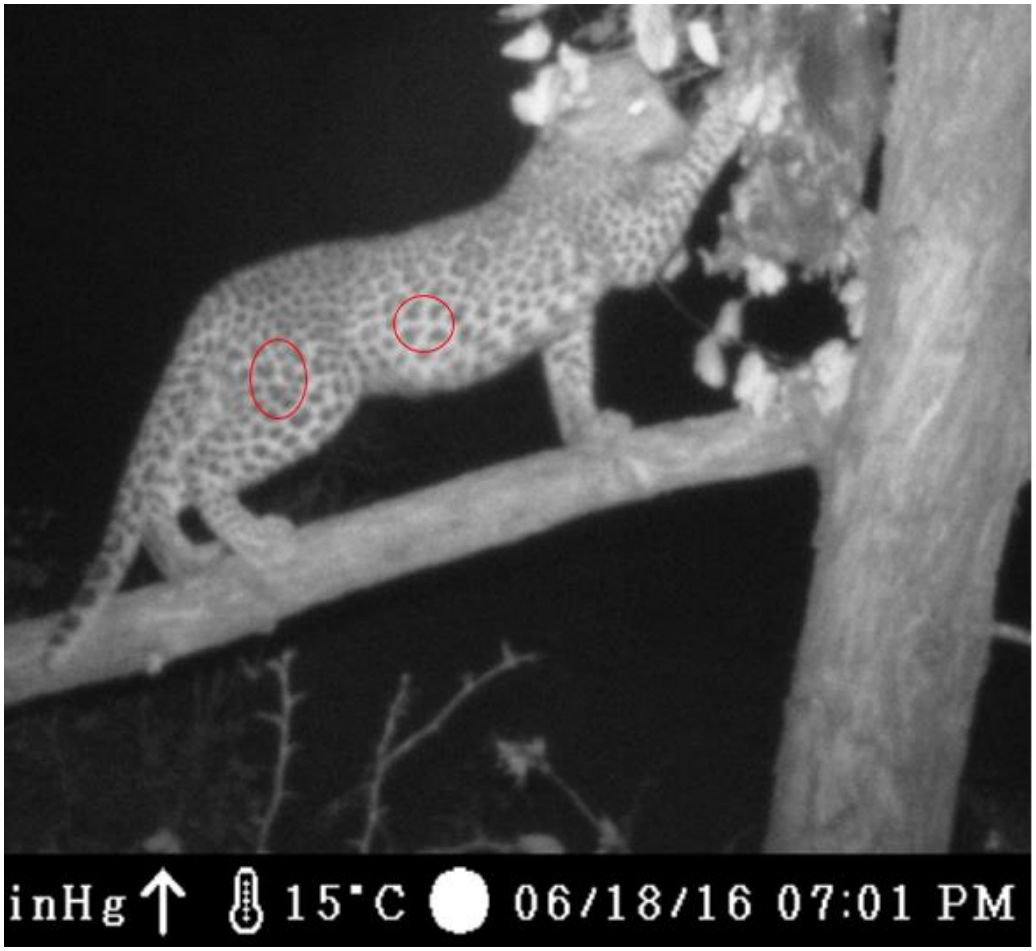
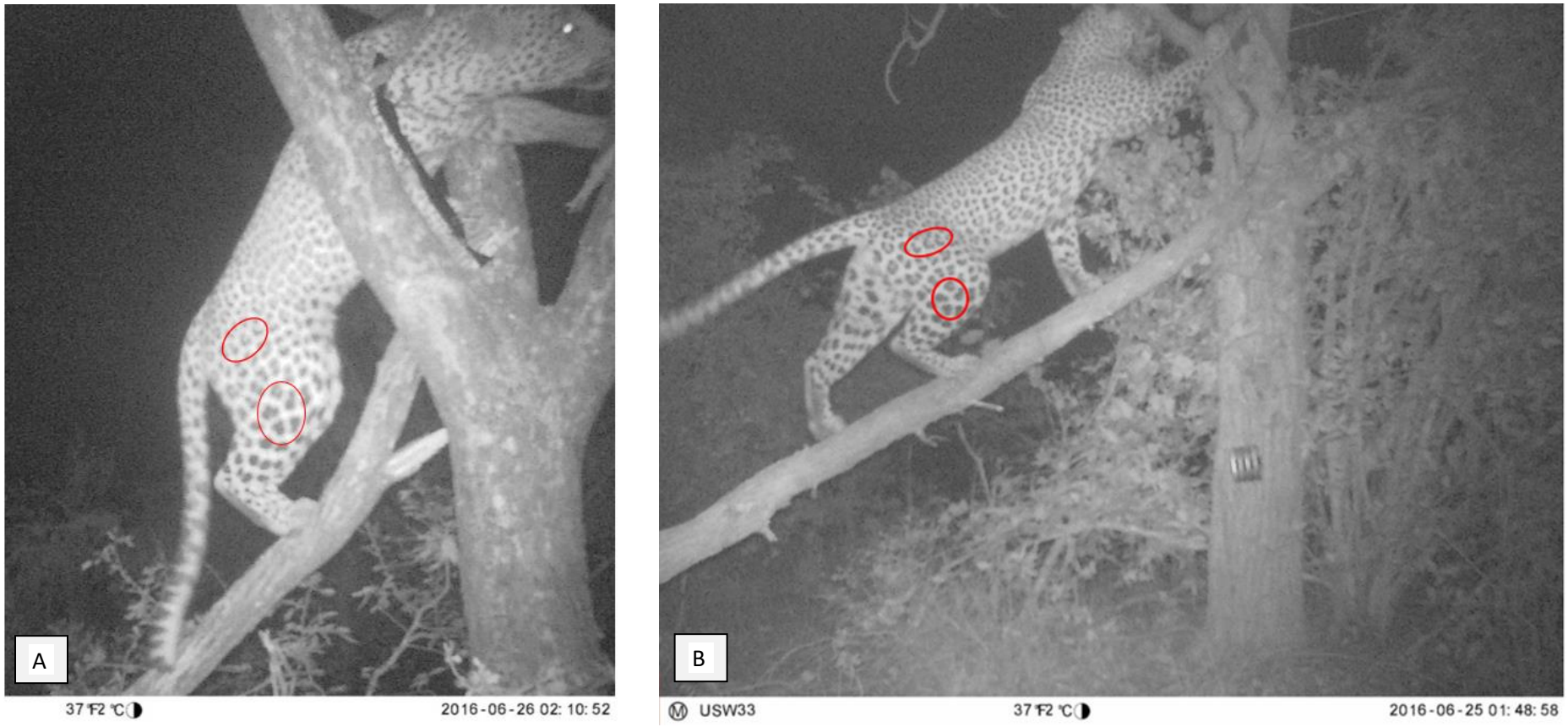
Appendix IX. *Panthera pardus pardus* Identification

Individual L1. Location - Bait trap 1, Wildebeest Way. First of two African leopard (*Panthera pardus pardus*) individuals identified by camera trap data. Hair sample collected. Identifiable by non-functioning radio collar (circled), leopard named by Selati Game Reserve as Cleo (0:1). Since collar failure (March 2015) no observations or camera trap data had been collected of Cleo. As such, according to reserve management and field guides, this data provides the first visual of the individual since that time. Photo enhanced to make markings more clear for pattern detection.

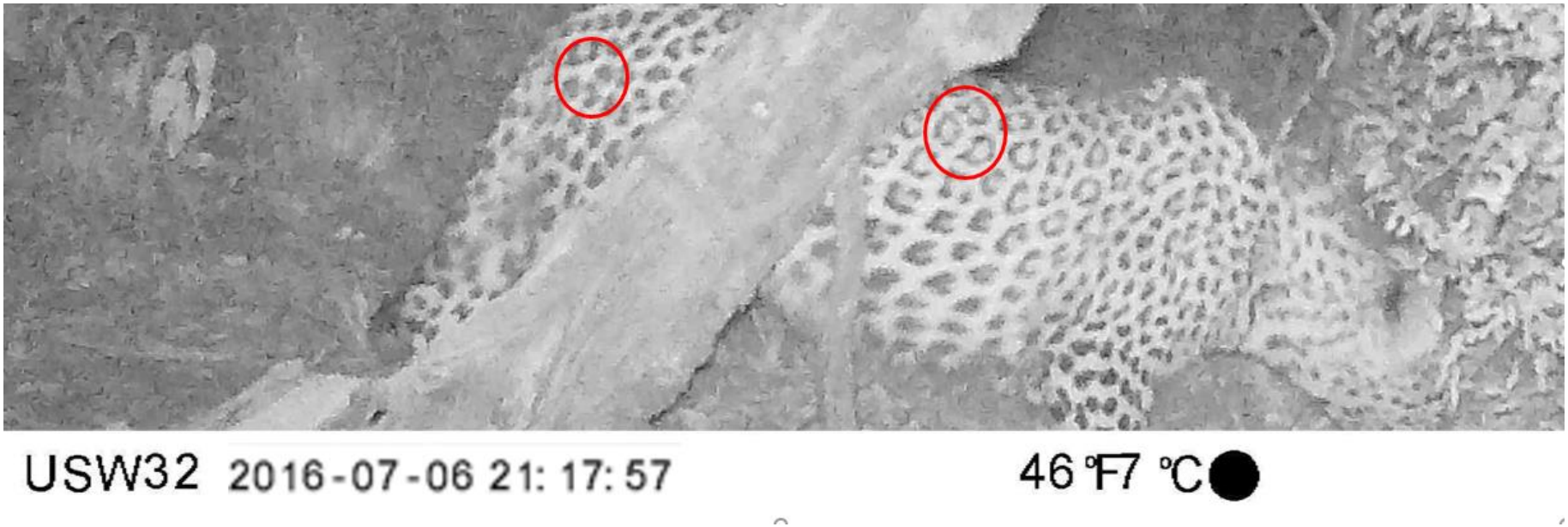


Individual L2. Location - Bait trap 1, Wildebeest Way. Second of two African leopard (*Panthera pardus pardus*) individuals identified by camera trap. Presence of both L1 and L2 in same time period imply L2 is the juvenile offspring (0:0:1) of L1. Hair sample collected. Identifiable rosette markings of right flank circled. Absence of radio collar and small size used to distinguish between the two individuals that occurred in the same time period.



Individual L3. (A) Location - Bait trap 2, Thankerton Cutline. African leopard (*Panthera pardus pardus*) presence (1:0), hair sample collected. Identifiable rosette markings of right flank circled. (B) Location - Bait trap 4, Burren Koppie. Identifiable rosette markings of right flank circled and matched to that of L3, hair sample collected.

Individual L4. Location - Bait trap 3, Steve's Cutline. African leopard (*Panthera pardus pardus*) presence (1:0), hair sample collected. Identifiable rosette markings of right flank circled.



Individual L5. Location - Bait trap 5, Jacket Plum. African leopard (*Panthera pardus pardus*) presence (0:0:0:1), but no in-tree bait trap inference. No sample collected. Identifiable rosette markings of right flank circled.

Individual L6. Location - Bait trap 5, Jacket Plum. African leopard (*Panthera pardus pardus*) presence (0:1), hair sample collected. Identifiable rosette markings of right flank circled. Photo enhanced to make markings more clear for pattern detection.

Individual L7. Location - Bait trap 6, Salejan. African leopard (*Panthera pardus pardus*) presence (1:0), hair sample collected. Individual unidentifiable and incomparable by phenotype to the other identified individuals.