PCR-high resolution melting analysis of the *COI*, *cyt b* and *16S rRNA* mini-barcode genes: a tool for species identification and discrimination in illegal bushmeat trade

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A dissertation Submitted in partial fulfilment of the requirements for the Degree of Master of Science in Molecular Biology and Bioinformatics of Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This research thesis is my original work and has not been presented elsewhere for an award of any degree

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DEDICATION

To dad and mum, Mr. Absalom Ouso Sunga and Mrs. Roselyn Ayoo Okuku, and all my siblings for the unwavering support through my studies.

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine Triphosphate
BEAN	Bushmeat-free Eastern Africa Network
BOLD	Barcode Of Life Database
bp	base pairs
BWP	Barcode of Wildlife Project
CBOL	Consortium of Barcode Of Life
CITES	Convention on International Trade in Endangered Species
CLS	Cell Lysis Solution
COI	Cytochrome *c* Oxidase I
cyt b	Cytochrome b
dF	Change in fluorescence
dT	Change in time
DNA	Deoxyribonucleic Acid
dNTPs	DiNucleotide TriPhosphates
dPCR	Direct PCR
dsDNA	Double Strand DNA
dT	Change in Time

EDTA	Ethylenediaminetetraacetic acid
GC	Guanine, Cytosine
GCF	Giraffe Conservation Foundation
GDP	Gross Domestic Product
HRM	High Resolution Melt(ing)
HRMA	High Resolution Melting Analysis
HWC	Human Wildlife Conflict
INTERPOL	International Police
IUCN	International Union of Conservation of Nature
IWT	International Wildlife Trade
KEBS	Kenya Bureau of Standards
KWS	Kenya Wildlife Service
MOPC™	Macrogen Oligonucleotide Purification Cartridge
MSA	Multiple Sequence Alignment
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NHP	Non Human Primate
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA

RCF	Relative Centrifugal Force
RFLP	Random Fragment Length Polymorphism
RGQ	Rotor-Gene Q
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulphate
SNPs	Single Nucleotide Polymorphism
STR	Sequence Tag Repeats
tRNA	Transfer RNA
US$	United State Dollars
WSPA	World Society for the Protection of Animals

ABSTRACT

Reliable molecular identification of vertebrate species from morphologically unidentifiable tissue is critical to the prosecution of illegally traded wildlife products to limit their trade, as well us for surveillance to inform conservation policies and identification of blood-meal hosts. Currently, this is mainly dependent on sequencing of the cytochrome c oxidase subunit I (*COI*) 'barcode' genes, which remains costly for purposes of screening large numbers of unknown samples and routine surveillance. High-resolution melting (HRM) analysis was optimized as a cheaper supplement to conventional sequencing. Here, we adopted HRM analysis of *COI*, *cytochrome b* and *16S ribosomal RNA* mini-barcode genes Polymerase chain reaction products. The process uses DNA intercalating dye to report the unique melting pattern of duplex DNA. We analysed 107 samples using the optimised approach and robustly differentiate 10 domestic species from 24 wildlife species that are common in the East African illegal wildlife trade. To validate the tool, we assessed whether bushmeat was sold in township butcheries by covert field surveillance sampling in Naivasha, Kenya. We identified one out of 90 samples as bushmeat (giraffe). This approach is being adopted for high-throughput pre-screening of potential bushmeat samples for exclusion of non-bushmeat samples from downstream processing in forensic species identification pipelines in Kenya and Tanzania. It is also useful as a sustainable surveillance and monitoring tool for illegal bushmeat trade, as well as for studies on hematophagous invertebrate vector-host interactions at wildlife-human interfaces in disease epidemiology.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Unsustainable hunting, consumption and sale of bushmeat in Africa contribute immensely to global bushmeat trade, which is valued at several billion dollars. Up to 270 tonnes of bushmeat were flown into Europe through a single airport in 2010 (Chaber et al., 2010) from Africa. While it is a crisis for wildlife in central and western Africa, it is a growing problem in East and Southern Africa (Barnett, 1997). Efforts to regulate or prevent the trade depend, among other factors, on an accurate and efficient identification of species for confiscated and surveillance samples. Currently the ability to efficiently do so is unsatisfactory.

The trade is mainly fuelled by the demand for consumption and income supplementation (Cawthorn & Hoffman, 2015). The consequences of direct human associations with some wild species have been severe. For instance Ebola virus disease (EVD) is a fatal disease that infect humans upon contact with infected wild animals such as fruit bats, non-human primates and forest antelopes (Judson et al., 2016; Leroy et al., 2004). The impact of bushmeat hunting on animal populations can also be severe. Many favoured species for bushmeat are already endangered, some close to extinction (Sollund & Maher, 2015) and there are flow-on effects for forest ecosystems (Wilkie et al., 2002) and tourism.

A number of efforts have been put in place to limit trade in our flora and fauna. These include awareness campaigns, fencing of parks, and conservancies among others. However, cases of bushmeat hunting are still rampant even with laws against such activities, thus there is need of better law enforcement strategies. But law enforcement can only be effective when backed by solid surveillance, policies and by efficient prosecution, which consequently relies on concrete evidence and informed policies. Accurate identification of confiscated species forms the basis of evidence, which is currently generated in a costly way, by long cytochrome c oxidase subunit I (*COI*) sequencing as the preferred standard (Dawnay et al., 2007; Hebert et al., 2003).

Molecular markers for DNA analysis have replaced earlier methods of identification. They include restriction fragment length polymorphism (RFLP) (Bing & Bieber, 2001), random amplification of polymorphic DNA (RAPD) (Partis & Wells, 1996) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995). However, these suffer poor reproducibility and development of reference databases is not possible (Campbell et al., 2003). Also, results from a mixed samples may be difficult to interpret due to overlapping amplification and restriction patterns (Maškova & Paulíčková, 2006). More recently, sequence tag repeats (STRs) (Nicklas et al., 2012) and single nucleotide polymorphism (SNPs) (Reed & Wittwer, 2004) have been used, which are however limited to detecting very specific wildlife species. The current gold standard is the sequencing of the long mitochondrial *COI* gene. Generally mitochondrial DNA (mtDNA) is preferred to nuclear DNA (nDNA) in species identification (Pereira et al., 2012).

High-resolution melting analysis (HRMA) is a fast, sensitive and specific tool developed for the detection of sequence variants and genotyping (Wittwer, et al., 2003). It employs the use of intercalating fluorescent dyes, such as SYBR Green I (Adaszek & Winiarczyk, 2010), or dsDNA-binding dyes such as EvaGreen (Mao et al., 2007), among others, to capture data on the precise melting of amplicons. The dyes undergo rapid solvent quenching as the duplex DNA is melted. The amplicon melting temperature (Tm) and specific shape of the melt curve is dependent on DNA complementarity, G-C content, and amplicon length (Wittwer, 2009), which we relied on to test species characterization instead of sequencing. While HRMA has been used with a number of genes for identification of species in viruses (Villinger et al., 2017), bacteria (Li et al., 2012), insects (Ajamma et al., 2016), plant products (Ganopoulos et al., 2013), animals (Naue et al., 2014), it has not been used with *COI*, *cyt b* or *16S rRNA* markers across a wide range of vertebrate species to aid forensic pipelines for identifying illegally traded wildlife products.

This study sought to evaluate the feasibility of HRMA of mitochondrial *COI*, *cyt b*, and 16S gene PCR amplicons for species identification across vertebrate species commonly targeted for bushmeat in East Africa (Stella et al., 2012) and also to differentiate them from common domestic species (cattle, goats, sheep, swine, chicken).

1.2 Problem Statement

A repertoire of risk is associated with the trade and consumption of bushmeat, from biodiversity conservation to human health. Most target species for bushmeat poaching are already endangered, some close to extinction. Bushmeat poaching perturbs the feeding

hierarchy, so that the number of large prey animals will reduce due to prey scarcity. Also the forest composition, structure and biomass is bound to irreversibly change as animals that disperse up to 80% of tree seeds are extinct. Some of the species hunted and consumed have been involved in the most damaging zoonoses, such Ebola virus disease. In East Africa, tourism is one of the major contributors to the respective country's GDP, therefore a decline in the number of tourist-attracting species threatens this sector with far reaching effects. Despite these, surveillance as one of the tools for deterring poaching has not been effectively utilized, especially in pursuing sustainable means for identifying species in surveillance exercises or forensic evidence generation pipelines.

1.3 Justification

Species identification of a sample, whether whole animal or part of it or even its products in different forms, is critical in curbing the International Wildlife Trade (IWT) and must be defined. Wildlife samples have been identified by three main approaches, which remain unsatisfactory for routine surveillance or high-throughput assays.

Firstly, morphological identification which has a limited degree of confidence, high level of expertise is required here to reference databases, while identifying feature may be intentionally removed. Secondly, biochemical markers like serological antibody-antigen reactions such as ouchterlony test can be used to identify tissues. But it is laborious and requires large amounts of sample. Moreover, most proteins lose their native functions soon after the death of the animal. The third approach utilises molecular markers for DNA analysis. These have historically included restriction fragment length polymorphism (RFLP), random

amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analyses. However, these techniques are obsolete since they suffer irreproducibility and development of reference databases is not possible. Moreover, recently, sequence tag repeats (STRs) and single nucleotide polymorphism (SNPs) have been used, which are however limited to detecting very specific wildlife species. All this favour the proliferation of illegal bushmeat use, rather than helping in efforts to deter it. This justifies the need of an alternative approach to species identification, that does not suffer the disadvantages of the above approaches. HRM analysis, being quicker and cheaper than the afore mentioned methods was adopted as a sustainable method in species identification pipelines.

1.4 Hypothesis/research questions

1. Can *COI*, *cyt b* and *16S ribosomal (r)RNA* mini-barcode genes HRMA be useful for bushmeat species identification?
2. How does the species identification resolution of *COI*, *cyt b* and *16S ribosomal (r)RNA* mini-barcode genes HRMA compare?
3. Can *COI*, *cyt b* and *16S ribosomal (r)RNA* mini-barcode genes HRMA used to differentiate domestic from wild animal species?

1.5 Objectives

1.5.1 General objective

To evaluate PCR-HRMA for bushmeat species identification using three mtDNA genes.

1.5.2 Specific Objectives

1. To optimize high-resolution melting analysis (HRMA) based on *COI*, *cyt b* and *16S ribosomal (r)RNA mini-barcode* genes for bushmeat species identification.
2. To compare the species identification resolution of *COI, cyt b* and *16S ribosomal (r)RNA* genes HRMA.
3. To validate the three-gene HRMA for species identification.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Techniques used in the identification of bushmeat

Wildlife forensic science uses scientific procedures to examine, identify, verify and compare evidence from crime scenes, and further relate the evidence to victims, animals, and suspects. Species identification of a sample, whether whole animal or part of it or even its products in different forms, is critical in curbing the IWT and must be defined. Wildlife samples have been identified by three main approaches.

Firstly, morphological identification is one of the earliest forms of identification, which relies on the observable features such as whole skin and skeleton, followed by anatomy, microscopic and osteological analysis to identify sample with a certain degree of confidence (Martiniakova et al., 2006). However, a high level of expertise is required, apart from identifying features being intentionally removed by suspects (Bartlett & Davidson, 1992) making morphological approaches unsuitable (Verma et al., 2003).

The second alternative is using biochemical markers like serological antibody-antigen reactions such as ouchterlony test can be used to identify tissues. This method has been applied by World Society for the Protection of Animals (WSPA) to developed an immunoassay for detection of bear protein in products suspected to have biological material from a bear species (Peppin et al., 2009). However, the flip side of this technique is that the

process of producing and isolating species-specific antibodies is laborious process (Macedo-Silva et al., 2000) and requires large amounts of sample. Moreover, most proteins lose their native functions soon after death of the animal (Bartlett & Davidson, 1992).

The third approach to sample identification utilises molecular markers for DNA analysis. These have historically included restriction fragment length polymorphism (RFLP) (Bing & Bieber, 2001), random amplification of polymorphic DNA (RAPD) (Partis & Wells, 1996) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995) analyses. However, these techniques are currently considered obsolete since they suffer irreproducibility and development of reference databases is not possible (Campbell et al., 2003). Also, results from a mixed samples may be difficult to interpret due to overlapping amplification and restriction patterns (Maškova & Paulíčková, 2006). More recently, sequence tag repeats (STRs) (Nicklas et al., 2012) and single nucleotide polymorphism (SNPs) have been used, which are however limited to detecting very specific wildlife species (Reed & Wittwer, 2004).

2.2 Mitochondrial DNA (mtDNA) for species identification

Mammalian mitochondrial DNA is a closed-circular double stranded DNA found inside the mitochondria, the cell energy factory for almost all cellular processes (Karnkowska et al., 2016). The human mtDNA genome was the first of mitochondrial genomes to be fully sequenced (Anderson et al., 1981). The mitochondrial genome's organisation and structure is quite conserved within mammals (Boore, 1999). The size of the mtDNA in humans is 16,592 nucleotides with 13 genes, 2 rRNAs and 22 tRNAs. In plants the mtDNA is much bigger, 180 to 720 Kbp, due to the occurrence of pseudo genes and introns. Arabidopsis mtDNA is

366,924 nucleotides coding for 57 identified genes, which is only 10% of the genome (Unseld et al., 1997). The duplex consist of two strands, the heavy (H) and light (L) chains, inferred on the basis of G-C base composition which results in distinct separation in a denaturing caesium chloride density gradient (Pereira et al., 2012). **Figure 2.1** below illustrates the genome organization of the human mitochondrial genome (Strachan & Read, 2010). Specific characteristics make mtDNA ideal for species identification (Panday et al., 2014); there are hundreds of copies of mtDNA per cell (Legros et al., 2004); mtDNA is protected from degradation due to its compartmentalization within a rigid high-protein mitochondrial membrane (it can be found in degraded samples such as bone and air where nDNA would be degraded) (Gray, 1989); the DNA Polymerase γ, which replicates mammalian mtDNA has an intrinsic 3′→5′ exonuclease activity (apart from its 5′→3′ polymerase activity), is highly mispair-specific and ensures high fidelity replication with fewer changes over time (Cotterill & Kearsey, 1991); and maternal inheritance means that all maternal lineages will have the same mtDNA (Rastogi et al., 2007). Furthermore, its closed circular organization hinders enzymatic degradation due to lack of exposed ends (Pereira et al., 2012). Most molecular markers described above target mainly the nuclear DNA (nDNA) in their analysis. However, forensic species identification currently favours genetic loci in mtDNA (Nelson & Melton, 2007).

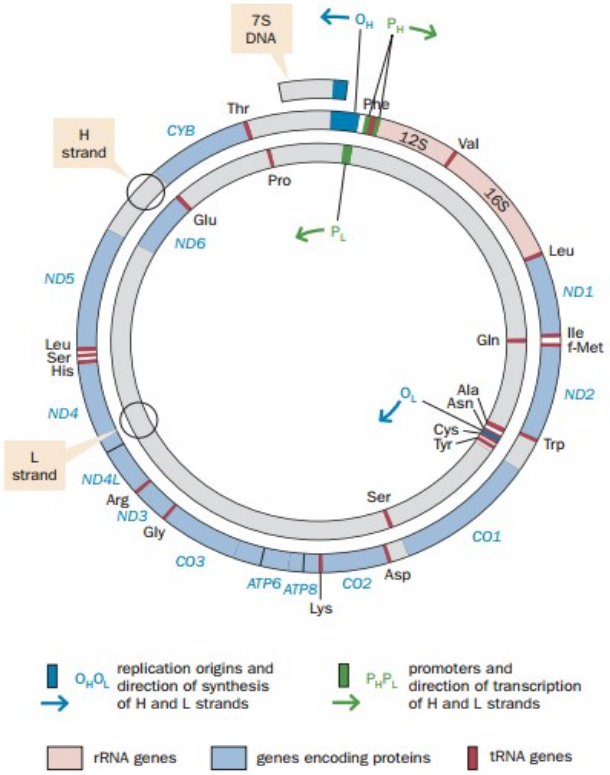

Figure 2.1: The organization of the human mitochondrial genome showing COI, cyt b and 16S genes (Strachan and Read, 2011).

2.3 International Wildlife Trade (IWT)

An alarming rise in illegal trade involving "the wild" is of high global concern currently, as captured in the wildlife trade policy reviews of the United Nations' Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2010). Illegal trade in wildlife ranks third largest illegal trade in the world after drug trafficking and weapons smuggling (Giovanini, 2006). A joint study by the United Nations Environmental Programme and INTERPOL indicates that different sources estimate the illegal trade in fauna and flora, other than fish and timber, to be well worth US$ 7 to US$ 23 billion yearly (Nellemann et al., 2014). Within these estimates, the global illegal bushmeat trade is valued at

US$ 1 billion. However, this figure may be higher considering the concealed nature of the trade, lack of effective surveillance and the lack of proper record keeping.

Trafficking of illegal wildlife and their products has been found to use similar strategies as drug trafficking (Wyler & Sheikh, 2008). The international wildlife trade (IWT) has become increasingly organized, with structured poaching, use of threat of violence, is well armed, non trivial financial support, international management of shipments and sophisticated forgery and counterfeiting of permits (Cowdrey, 2002). The highest profile IWT involves elephant ivory, rhino horns and tiger products, with the main markets being in Asia. It has been estimated that nearly 25,000 elephants were killed in 2013 for ivory supply, a number well correlating with the report of a kilogram of ivory costing US$ 2200 in the streets of Beijing (Park, 2014). By December 2018, the Elephant protection initiative estimated that 20,000 elephants are killed annually, translating to 55 per day in Africa. The data for rhinoceros are not pleasing neither, with 1000 slaughtered in the same period in South Africa. The rhinoceros horn, with its purported medicinal value, is thought to cost US$ 6600 per kilogram in the black market (Park, 2014). Over the century, tiger population has fallen from 100,000 to less than 3,500 today. Illegal poaching accounts for up to 78% of Sumatran tigers (Sollund & Maher, 2015).

2.4 Bushmeat in Africa

Bush is a general term used to refer to the forest and thicket in Africa. Therefore, “bushmeat” is coined to mean meat found from the bush, the wild included. It applies to all wildlife species targeted for human consumption and/or valuable parts, including elephant, gorilla, chimpanzee and other primates, forest antelope (duikers), porcupine, bush pig, cane rat,

pangolin, monitor lizard, guinea fowl, among others (Nellemannet al., 2014). In the early 20th century, Africa was estimated to have a million black rhinos belonging to four sub-species. However, by 2007 it was feared that the Western black rhino, with a distinct dexterous upper hip, had become extinct, the number of wild northern white rhino also shrunk like never before (Park, 2014). Incidences of poaching and illegal trade in ivory, which had reduced in the 1990s, began to rise again with an unprecedented spike in illegal trade of elephant tusks and rhinoceros horns (Park, 2014).

The bushmeat trade in Africa, vis a vis the rest of the world, is mainly driven by the need for meat as a cheap (in the rural set ups) diet protein supplement. It is a delicacy for the rich in urban populations, which makes it costly than domestic meats.. A study in Switzerland, identified that 91% confiscated bushmeat originated from Africa, with Kenyan wildlife representing 7% of the trade (Wood et al., 2014) (**Figure 2.2**). Chaber and others estimated the amount of bushmeat exported from African to Europe to be 270 tonnes (Chaber et al., 2010). The population of the world continues to rise, with Africa projected to have a population of 1.186 billion people in 2015 and 2.478 billion by 2030 (Bash, 2015). Pressure is bound to increase on encroachment into African bush lands. Hunting wildlife is an ancient practice through the history of humans, which has persisted to this day (Winterhalder & Kennett, 2006). In West Central Africa, up to 40% of the rural poor depend on bushmeat for protein, while only 4% bushmeat is served in urban diets where chicken is quite cheap.

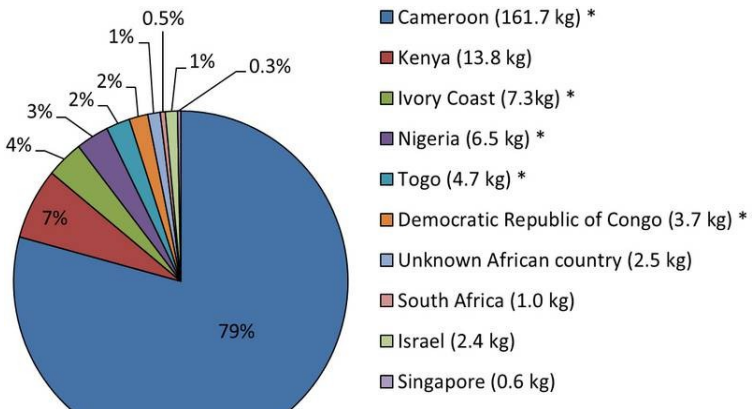

Figure 2.2: The origin of bushmeat confiscated in Switzerland (Wood et al., 2014).

2.5 Bushmeat in Kenya

Like in many countries in Africa, illegal dealing in wildlife in Kenya has also evolved, leading to new challenges to wildlife conservation. Thirty-three thousand elephants and 1,010 rhinos estimated to be in Kenya, plus an additional plethora of other wildlife, are scattered beyond the national parks to conservancies, communal and private land, sanctuaries, council territories and private ranches (UN Chronicles: Illegal Wildlife Trade, Fighting Wildlife Trade in Kenya. Vol. LI No. 2, 2014). Previously, Kenya experienced high levels of poaching of elephants and rhinos, before the establishment of the Kenya Wildlife Services (KWS) in 1989. Once KWS was established, and due to the international ban on ivory trade by CITES in the same year, poaching slowed, but continues to be a threat to populations. Unfortunately, there has been resurgence, in recent years, of widespread poaching and trafficking, with new challenges (Adetunji, 2008; CITES et al., 2013). Poachers have devised new methods (poisoning and snaring, in place of firearms) and targeted new territories. If rates of poaching persist, local African elephant populations could disappear by the next decade, according to estimates by AWT, 2014. Within two decades, between 1977 and 1997, there was a wildlife

decline of 38% in all areas surveyed nationally and by 36% in protected areas (Iregi & Heather, 2009). More recent data on wildlife decline in Kenya rangelands is severe, (Ogutu et al., 2016) report up to 68% decline between 1977 and 2016.

There are seven "Bushmeat Consumption Hotspots" in Kenya, namely Machakos/Kajiado, Taita/Taveta, Kitui/Mwingi, Trans Mara, Laikipia, Meru/Tharaka and Tana River/Malindi (Iregi & Heather, 2009). These are wildlife rich areas close to key protected areas, such as national parks, or within game ranches characterized by high levels of poverty and landlessness. It is largely unregulated and poses a significant threat to wildlife populations both inside and outside of protected areas. A single de-snaring team operating in a single area can lift 450 snares in two weeks of operations (Iregi & Heather, 2009). **Figure 2.3** below shows dik dik meat being prepared outside of Tsavo national park for urban markets (DSWT). Factors which have contributed to this include poor mechanisms for monitoring and information management on wildlife hunting and hampered trade informed discussions necessary to establish an improved wildlife policy with enabling legislation toward regulated consumptive use. Collaboration and engagement with the judiciary system has also intensified and further contributed to full enforcement of wildlife laws. Regionally, Kenya's cross-border collaboration with Tanzania and Uganda is targeting crimes of a trans-boundary nature and yielding results in combating illegal activities along shared borders. Kenya has been further supported by international and regional law enforcement bodies, such as INTERPOL and the Lusaka Agreement Task Force (LATF), which have been valuable in facilitating, coordinating and offering support with transnational crime investigations (UN Chronicles: Illegal Wildlife Trade, Fighting Wildlife Trade in Kenya. Vol. LI No. 2, 2014).

Figure 2.3: Dik dik meat being prepared for urban market outside the Tsavo (The David Sheldrick Wildlife Trust).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Samples

3.1.1 Samples for optimization

This were samples used to optimize and to generate reference HRMA profiles. Sample types included tissue and blood (**Table 3.1**) and were provided by the Kenya Wildlife Service (KWS) forensic laboratory. Some of the domestic species were bought from local supermarkets or butcheries. We targeted, among others, commonly hunted wildlife (Stella et al., 2012), and common domestic species (cattle, goat, sheep, donkey, pig, camel, rabbit, turkey chicken and cat). KWS meat samples exhibited varied levels of integrity depending on their state at the point of confiscation. After confiscation or sampling, meat and blood had been stored in -40°C freezers in the KWS forensic laboratory. Hide were stored at room temperature. Samples were grouped mainly according to their taxonomic families, including Bovidae, Camelidae, Cheloniidae, Elephantidae, Equidae, Felidae, Giraffidae, Leporidae, Phasianidae, Rhinocerotidae and Suidae (**Table 3.1**). The numbers of species within each taxonomic family included in the study was limited by availability of samples.

Table 3.1: Source of the sample for species used in the HRM reference optimization.

Common name	Species	Family	Tissue	Blood	Total
Cow	*Bos taurus*	Bovidae	2	4	6
Goat	*Capra hircus*	Bovidae	1	1	2
Sheep	*Ovies aries*	Bovidae	1	1	2
Donkey	*Equus africanus asinus*	Equidae	1	0	1
Pig	*Sus scrofa*	Suidae	1	0	1
Camel	*Camelus dromedarius*	Camelidae	0	1	1
Rabbit	*Oryctolagus cuniculus*	Leporidae	1	0	1
Turkey	*Meleagris gallopavo*	Phasianidae	1	0	1
Chicken	*Gallus gallus*	Phasianidae	1	1	2
Domestic cat	*Felis catus*	Felidae	1	0	1
Waterbuck	*Kobus ellipsiprymnus*	Bovidae	3	0	3
African buffalo	*Syncerus caffer*	Bovidae	4	4	8
Impala	*Aepyceros melampus*	Bovidae	4	0	4
Grant's gazelle	*Nanger granti*	Bovidae	1	0	1
Common duiker	*Sylvicapra grimmia*	Bovidae	3	0	3
Blue wildebeest	*Connochaetes taurinus*	Bovidae	5	2	7
Hartebeest	*Alcelaphus buselaphus*	Bovidae	1	1	2
Sable antelope	*Hippotragus niger*	Bovidae	0	2	2
Eland	*Tragelaphus oryx*	Bovidae	2	0	2
Kirk's dik-dik	*Madoqua Kirkii*	Bovidae	1	0	1
Giraffe	*Giraffa camelopardalis*	Giraffidae	4	2	6
Bushbuck	*Tragelaphus scriptus*	Bovidae	1	2	3
Bushpig	*Potamochoerus porcus*	Suidae	2	0	2
Warthog	*Phacochoerus africanus*	Suidae	2	2	4
Chapman's zebra	*Equus burchellii chapmani*	Equidae	2	0	2
Grevyi's zebra	*Equus grevyi*	Equidae	0	2	2
Plain zebra	*Equus burchellii quagga*	Equidae	2	2	4
Savannah elephant	*Loxodonta africana africana*	Elephantidae	2	4	6
'Forest' elephant	*Loxodonta africana cyclotis*	Elephantidae	0	2	2
Black rhino	*Diceros bicornis*	Rhinocerotidae	0	4	4
White rhino	*Ceratotherium simus*	Rhinocerotidae	0	5	5
Lion	*Panthera leo*	Felidae	3	3	6
Leopard	*Panthera pardus*	Felidae	0	2	2
Cheetah	*Acinonyx jubatus*	Felidae	0	3	3
Green sea turtle	*Chelonia mydas*	Cheloniidae	4	0	4
Logger head sea turtle	*Caretta caretta*	Cheloniidae	1	0	1
		Totals	**57**	**50**	**107**

3.1.2 Samples for validation

This were samples used to validate the optimized HRM. Open butcheries were randomly sampled, between 10am and 4pm, in 22 mapped locations. Sampling was opportunistic. The areas were Kambi Daraja, Gilgil, Kinamba, Kasarani, Kihoto, Kamere, Kwa Muya, Kongoni, DCK, Ndabib, Duro, Kabati, Kanjoo estate, Mirema, Sanctuary, Karagita, Langalanga, Kikopey, Kambi Somali, Kongasis, Mutaita and Hell's Gate. Longonot and Mai Mahiu were sampled along the way. Samples from individual butcheries were separately packed and stored for sub-sampling later in the day. We sub-sampled each sample in triplicate into 1.8 ml cryovials, using a sterile scalpel and fresh gloves for every sample. The samples were stored in shippers with liquid nitrogen until extraction.

3.2 Genomic DNA extraction from tissue and blood

Genomic DNA was extracted from meat and blood using Qiagen's DNeasy Blood and Tissue Kit (Qiagen, Hannover, Germany) according to the manufacturer's instructions, with minimal modifications. Briefly, 3mm^3 of tissue (70µl of blood) was used as starting material in a 1.5ml micro-centrifuge tube with 180µl of Buffer ATL, followed by the addition of 25µl (20µl for blood) proteinase K, the homogenate was mixed by vortexing 3 X 10 seconds, and incubation done for 2 hours, blood samples were incubated for 1 hour in an eppendorf block mixer, with interval mixing every 3 sec. After vortexing for 15 sec, 200µl of Buffer AL was added into the lysate followed by 3-times-10-seconds vortexing for 10s three times. Incubation was done at 56°C for 2 hours. 200µl of 100% molecular grade ethanol was added to the mixture followed by vortexing. The resultant mixture was carefully pipetted into a DNeasy Mini spin column placed in a 2ml collection tube. Centrifugation was done at 12 000 x *g* (14200RCF) for 1 min

in a 5417R eppendorf bench-top fixed angle rotor centrifuge. The flow-through was discarded with the collection tube. The spin column was transferred into a new 2 ml collection tube. 500µl of buffer AW1 was added, followed by centrifugation for 1 min at 12000 x *g* (14200RCF). The flow-through was discarded with the collection tube. The spin column was transferred into a new 2 ml collection tube, 500µl of buffer AW2 was added, followed by centrifugation for 3 min at 20,000 x *g* (23600RCF). The flow-through was discarded with the collection tube. The spin column was transferred into a new 1.5 ml micro-centrifuge tube and DNA was eluted by adding 200µl of buffer AE to the centre of the spin column membrane, followed by incubation at room temperature (22°C) for 1 min. Centrifugation at 12000 x *g* (14200RCF) for 1 min completed the process. Extracted DNA was stored at -20°C until use.

3.3 Polymerase Chain Reaction with High Resolution Melting Analysis

Previously described primers targeting three vertebrate genes were used for PCR (**Table 3.2**). Primers were synthesised at 0.05µM scale and purified by MOPC™ method at Macrogen, Europe. The PCR master mix contained 2µl of pre-formulated 5X HOT FIREPol® EvaGreen® HRM Mix, no ROX (Solis BioDyne, Tartu, Estonia), Forward and reverse primers (Macrogen, Europe) were added to a final reaction concentration of 0.5µM and 2µl of template. PCR-grade water was used to bring the final reaction volume to 10µl. The PCR was performed in a HRM-capable thermo-cycler, RotorGene Q (QIAGEN, Hannover, Germany). Every run had a set of know control samples. The amplification cycling conditions included initial hold at 95°C for 15 minutes, then 40 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 20 seconds and extension at 72°C for 30 seconds. Final extension was done at 72°C for 5 minutes. Immediately after amplification, amplicons were gradually

melted at 0.1°C increments, with fluorescence acquisition every 2 seconds. Melting was done from 75°C to 95°C. From the melting, a graph of fluorescence against temperature (°C) was generated, which was then normalized between 0 and 100 fluorescence in the analysis. Every melting profile was observed against the profiles of the set of controls used, on a per-target basis. Samples presenting unique profiles were amplified using barcoding primers targeting a longer *COI* gene region.

Table 3.2: PCR-HRM primers.

Primer name	Sequence (5' to 3')	Target gene	Amplicon size (bp)	Reference
Uni-MinibarF1	TCCACTAATCACAARGATATTGGTAC	COI	~205	(Meusnier et al., 2008)
Ronping_R	TATCAGGGGCTCCGATTAT			(Lee et al., 2015)
Cyt b For	CCCCTCAGAATGATATTTGTCCTCA	cyt b	~383	(Boakye et al., 1999)
Cyt b Rev	CCATCCAACATCTCAGCATGATGAAA			
Vert 16S For	GAGAAGACCCTRTGGARCTT	*16S* rRNA	~200	(Omondi et al., 2015)
Vert 16S Rev	CGCTGTTATCCCTAGGGTA			

3.4 Conventional Polymerase chain reaction

Conventional PCR was used for confirmation of positive controls, samples with unique HRM profiles and validation, by long *COI* sequencing. The amplification was done in 10µl volumes comprising of 2µl of 5X HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia). Forward and reverse primers were added to a final concentration of 0.5µM and 2µl of template. PCR-grade water was used for top-up. We used previously described primers VF1d

(TCTCAACCAACCACAARGAYATYGG) and VR1d (TAGACTTCTGGGTGGCCRAARAAYCA) (Ivanova et al., 2012), for vertebrate barcoding based on the long (750bp) *COI* gene. Each primer was tagged with a M13 tail, TGTAAAACGACGGCCAGT and CAGGAAACAGCTATGAC, respectively, as adaptor for sequencing. Amplification conditions included initial hold at 95ºC for 15 minutes, then 40 cycles of denaturation at 95ºC for 60 seconds, annealing at 56ºC for 60 seconds and extension at 72ºC for 90 seconds. Final extension was done at 72ºC for 10 minutes. 2% agarose in 1X TAE buffer gel electrophoresis was done on amplicons before purification for sequencing. Amplicons with clear bands were purified using ExoSAP-IT (USB Corporation, Cleveland, OH), according to manufacturer's protocol. Sequencing was done at Macrogen, Europe.

3.5 Sequence Analysis

All sequences were queried, trimmed and analysed using Geneious v10.2.6 (available from http://www.geneious.com) software created by Biomatters (Kearse et al., 2012). The in-built BLAST option of Geneious was used to query GenBank and aligned sequences obtained with appropriate GenBank reference sequences. Complete mitochondrial genome reference sequences with the highest bit-score were used for the alignment. MUSCLE aligner option was used with default parameters to generate multiple sequence alignment (MSA) of reference and generated sequences.

3.6 Blind validation

To validate three-gene amplicon HRM analysis, we analysed 90 unknown covert samples through PCR-HRM. The samples were meat from a covert survey of butcheries in Naivasha (0°43' 0.01" N 36° 26' 9.28" E, about 77 km from the capital Nairobi), a town in Kenya. From

the resultant HRM profiles, we identified unknown samples by comparing their melting profile similarities (melting temperature and curve shape) to the profiles of already known controls. We further confirmed of the identities obtained by HRM analysis through sequencing of the long *COI* fragment and sequence alignment with GenBank reference sequences using Geneious software.

3.7 Data Analysis

The Rotor-Gene Q (RGQ) Software, provided with the machine, was used for HRMA. HRM curve was normalized for fluorescence between 0% and 100% to reduce the background noise using the two normalization regions, normalization region 1 (lower bound) and 2 (upper bound). Both the raw and normalized graphs plot fluorescence against temperature in degrees Celsius (˚C). Also, a melting curve plot of derivative fluorescence (dF/dT) against temperature (˚C) was generated. Species were identified and discriminated based virtual correspondence of unknown curve profiles to known control profiles.

CHAPTER FOUR

4.0 RESULTS

4.1 Species differentiation

HRM profiles were generated from short mitochondrial *CO1*, *cyt b*, and *16S rRNA* PCR products, using samples earlier described (**Table 3.2**), to differentiate and identify 10 domestic and 24 East African wild vertebrate species. Results is partly shown in **Figures 4.1 and 4.5** and is summarised in the matrix in **Figure 4.2**. Where possible, we had multiple samples for a single bushmeat species, while in other instances, there was only one reference sample. Species identifications were further confirmed by sequencing of the *CO1* barcode region. The domestic species, including three members of the Bovidae family (cattle-*Bos taurus*, goat-*Capra hircus*, and sheep-*Ovis aries*), one member each for Suidae (pig-*Sus scrofa*), Equidae (donkey-*Equus asinus*), Camelidae (camel-*Camelus dromedarius*), and Leporidae (rabbit-*Oryctolagus* sp.), and two Phasianidae (turkey-*Meleagris gallopavo* and chicken-*Gallus gallus*) were successfully differentiated from all other wild animal specimens by three-marker PCR-HRM analysis.

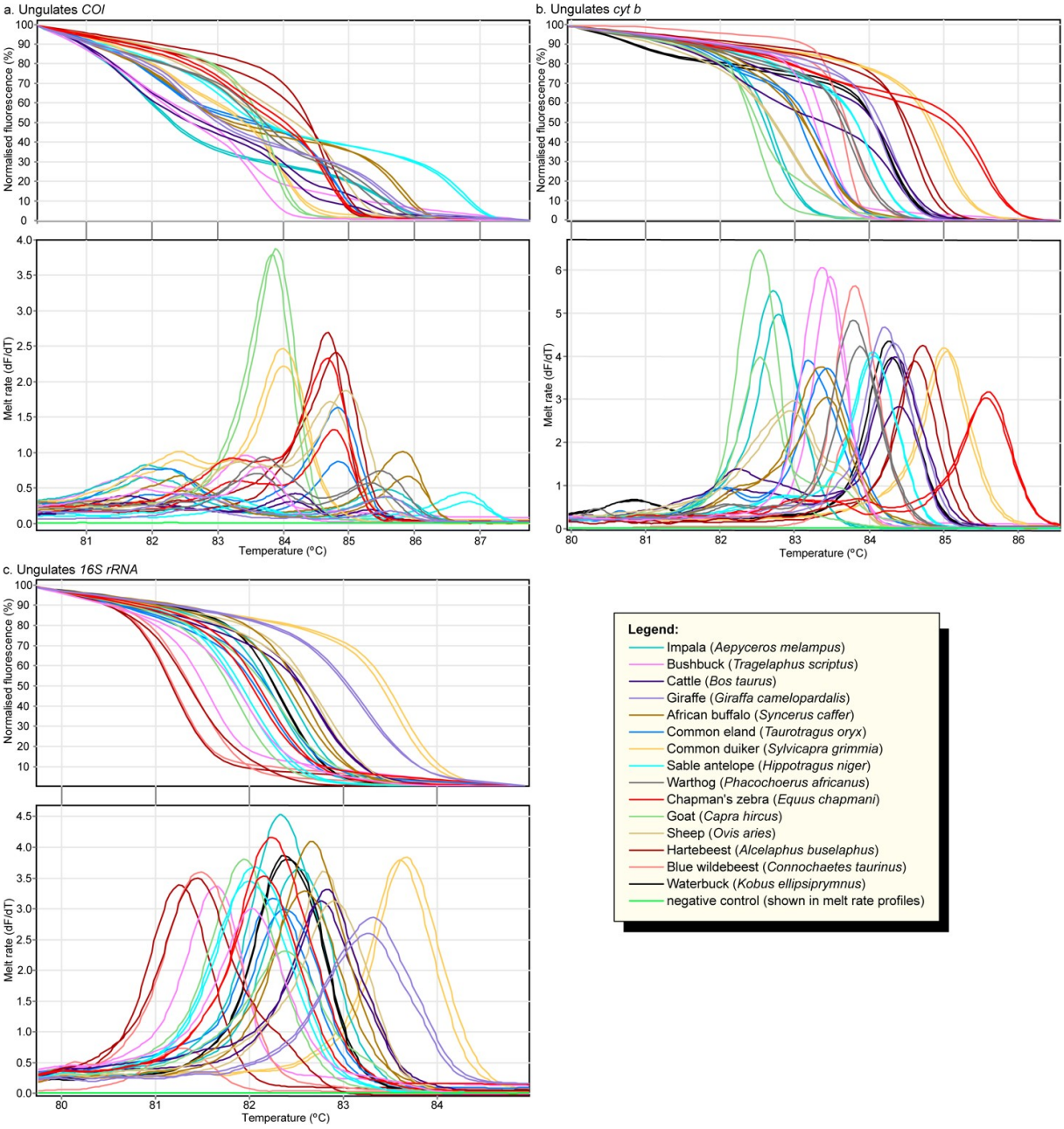

Figure 4.4: Distinct PCR-HRM profiles of ungulate species. Normalized HRM profiles are represented as percent fluorescence and melt rates are represented as change in fluorescence units with increasing temperatures (dF/dT) for (A) COI (B) cyt b, and (C) 16S rRNA markers.

Though most species could be differentiated by pair wise comparisons of HRM profiles using all three markers, some species could only be differentiated by one or two of the three markers, summarized in **Figure 4.2,** due to similar HRM profiles within 1°C melting

temperature (T_m) ranges or due to poor amplification of some species with the primers of particular markers. For example, waterbuck (*Kobus ellipsiprymnus*) failed to amplify with *COI*, but could be differentiated from all other species based on its *cyt b* and *16S rRNA* HRM profiles (**Figure 4.1**). Some species showed similarities in both shapes and melting temperature for particular markers. For example, among *COI* HRM profiles, pig samples generated a similar *COI* and *cyt b* HRM profiles within a 1°C T_m range to those generated by giraffe (*Giraffa camelopardalis*) samples, but could be clearly differentiated based on their distinct *16S rRNA* HRM profiles (**Figures 4.1 and 4.6**).

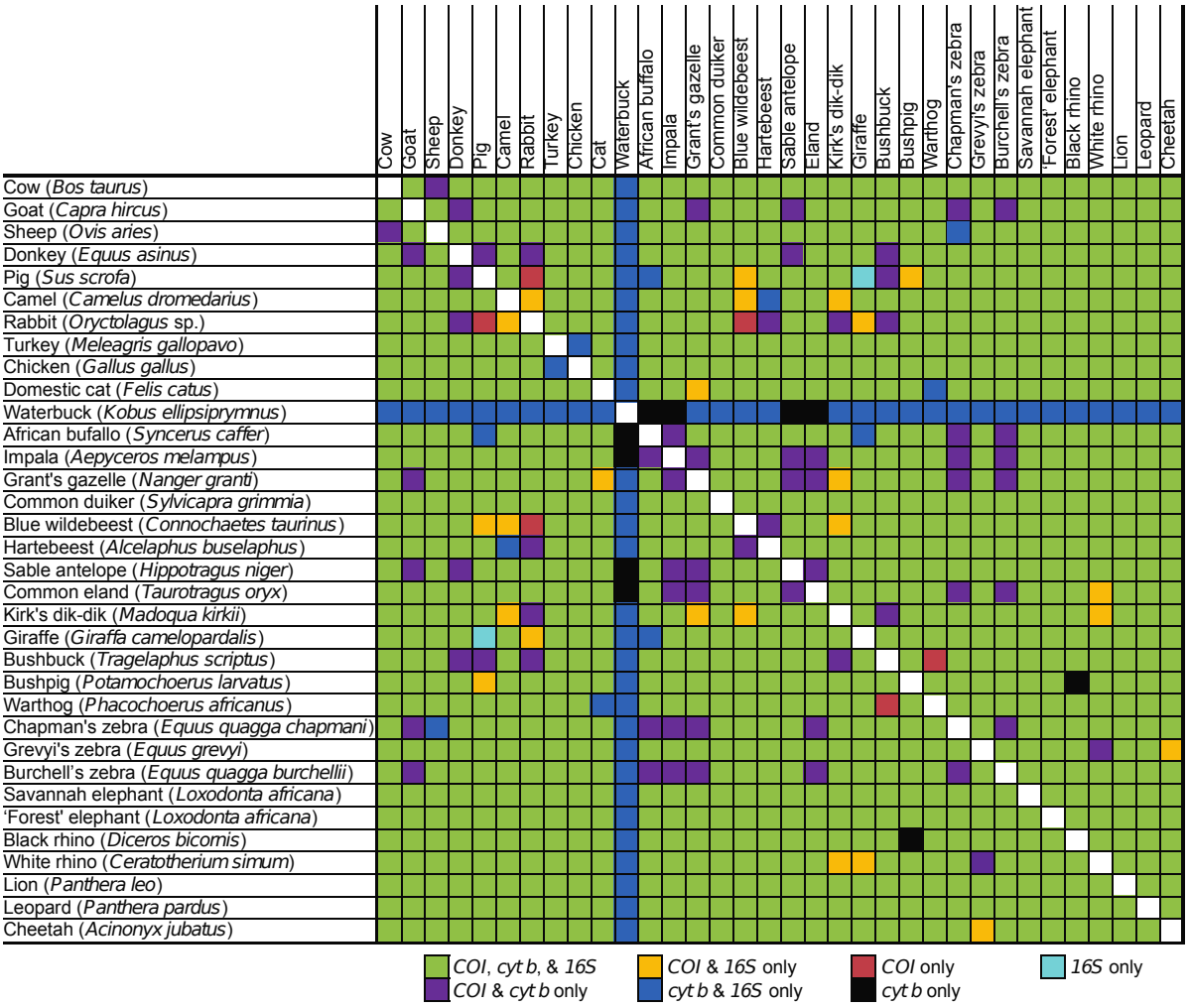

Figure 4.5: Summary matrix of pair-wise discriminations by PCR-HRM of 34 species and DNA marker resolution. Markers that generated distinct HRM profiles for pair-wise species comparisons are indicated by colours according to the legend.

The results show two distinct sets of HRM profiles for elephant reference samples obtained from Kenya Wildlife Service (KWS) using all three markers (**Figure 4.3**). Upon *COI* barcode sequencing of these samples, it was clear that one set of HRM profiles corresponded to the expected savannah elephant (*Loxodonta africana*), which is endemic to Kenya. Interestingly, the other set of HRM profiles were generated from savannah elephants with forest elephant (*Loxodonta cyclotis*) mitochondrial DNA (mtDNA), not endemic in Kenya. All markers were

also able to distinguish the two species of rhinos, black (*Diceros bicornis*) and white (*Ceratotherium simus*) rhinos (**Figure 4.3**). Among equine samples, zebra species endemic to East Africa (plains zebra-*Equus quagga*, Chapman's zebra-*Equus chapmani*, Grevyi's zebra-*Equus grevyi*) and donkey (**Figure 4.4**), as well as available Felidae reference samples (cheetah-*Acinonyx jubatus*, leopard-*Panthera pardus,* lion-*Panthera leo*, domestic cat-*Felis catus*) (**Figure 4.5**), could be clearly distinguished by three-marker PCR-HRM from each other and other domestic and bushmeat species, data summarized in (**Figures 4.2**). During early HRM experiments using DNA extracts provided by KWS, unique PCR-HRM profiles we obtained for loggerhead sea turtle (*Caretta caretta*) and green sea turtle (*Chelonia mydas*) **(Figure 4.6)**.

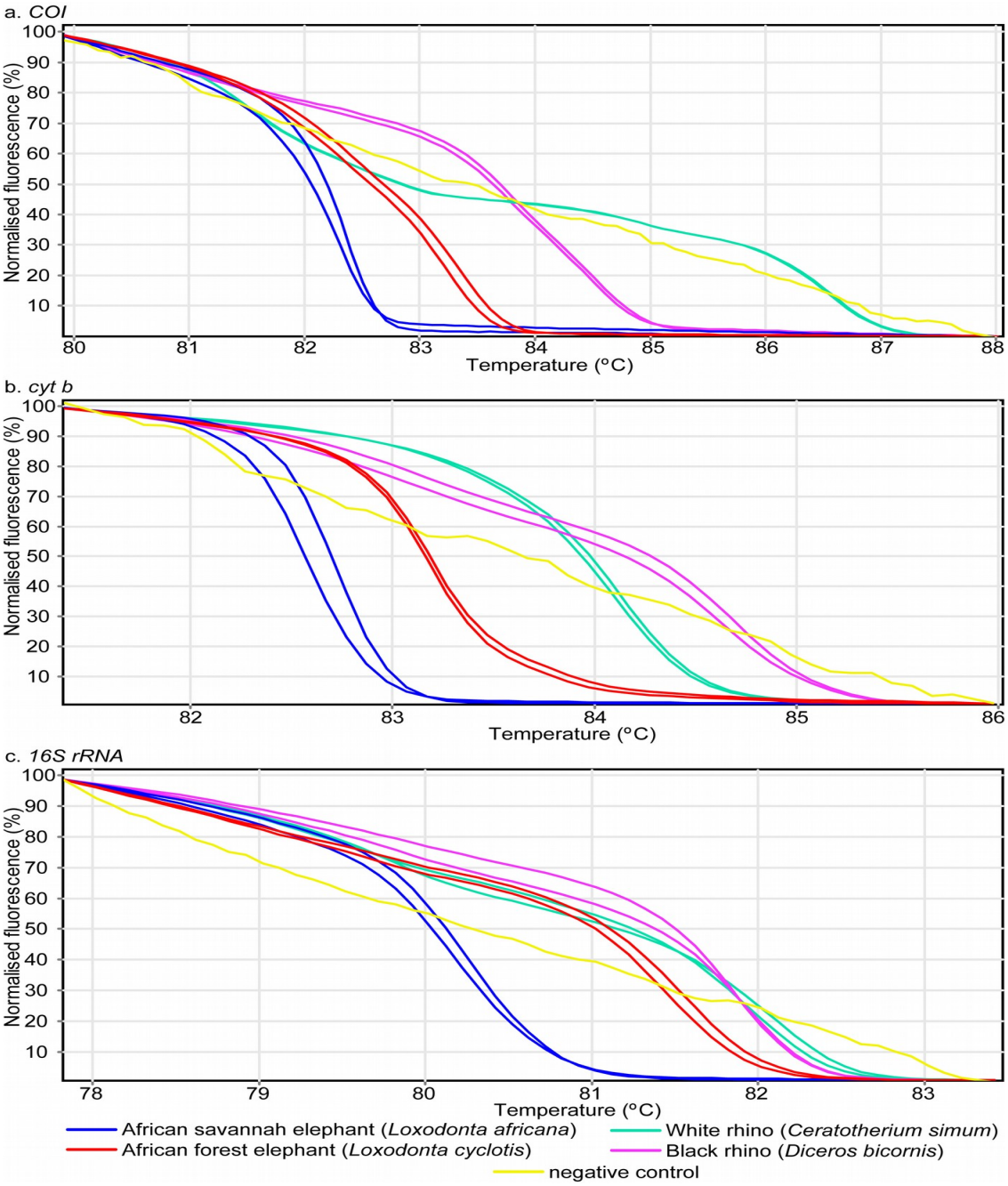

Figure 4.6: Distinct PCR-HRM profiles among Elephant and Rhino species. Normalized HRM profiles are represented as percent fluorescence for (A) COI (B) cyt b, and (C) 16S rRNA markers. The African forest elephant mitochondrial amplicons were obtained from African savannah elephant reference samples.

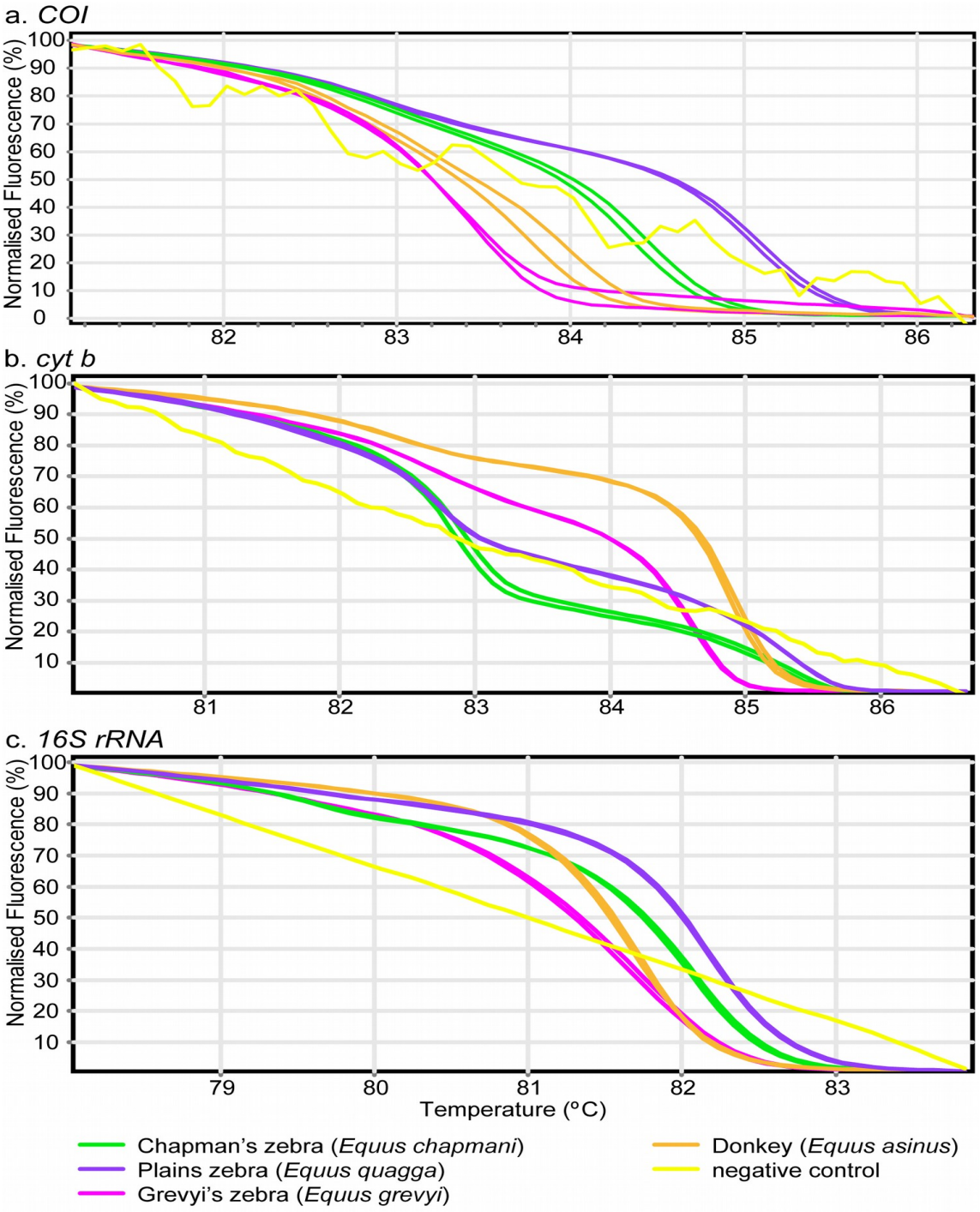

Figure 4.7: Distinct PCR-HRM profiles for the Equidae family showing the differentiation of three zebras sub-species and donkey. Normalized HRM profiles are represented as percent fluorescence for (A) COI (B) cyt b, and (C) 16S rRNA markers.

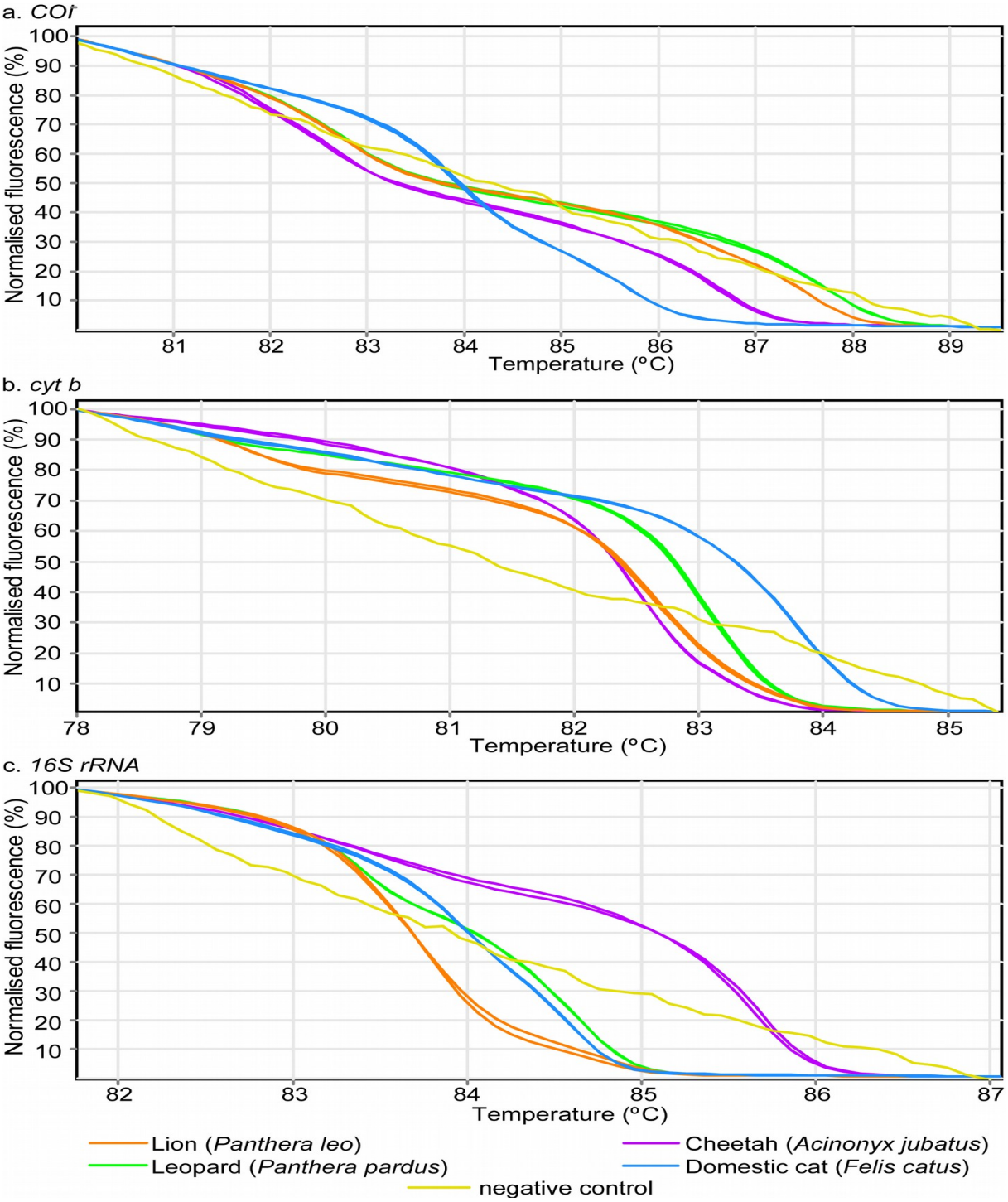

Figure 4.8: Distinct PCR-HRM profiles for Felidae family species. Normalized HRM profiles are represented as percent fluorescence for (A) COI (B) cyt b, and (C) 16S rRNA markers.

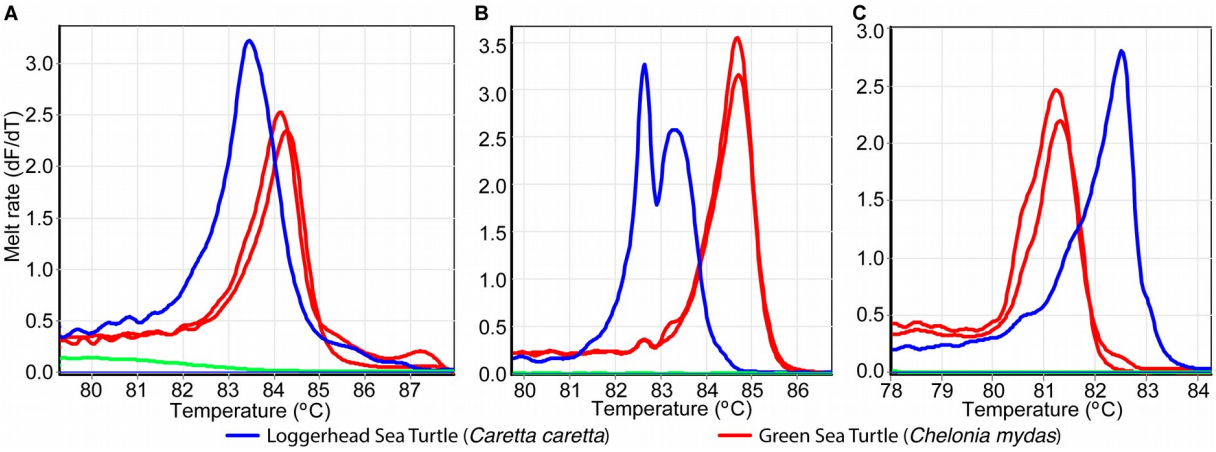

Figure 4.9: Distinct PCR-HRM melt profiles are represented as change in fluorescence units with increasing (dF/dT) for (A) COI (B) cyt b, and (C) 16S rRNA markers.

4.2 Marker discrimination comparison

From among the species tested, we did not encounter any that could not be distinguished from another by combined analysis of HRM profiles generated by all the three DNA markers. Out of 561 pair-wise comparisons (**Figure 4.2**), 39 pairs (7%) could not be distinguished by *COI* PCR-HRM, of which 33 pairs were due to non-amplification of a species during PCR, and 12 (2.3%) and 33 (6.3%) pairs could not be distinguished by *cyt b* and *16 rRNA* PCR-HRM, respectively. Although PCR-HRM analysis of the *COI* marker was consistently best at resolving species for DNA samples that amplified, giving unique melt curve profiles in shape and $T_{m.}$, the *cyt b* and *16S rRNA* markers had better PCR efficiency in all cases for any particular sample, observed by the lower C_T values and higher fluorescence values in the melt curve plot. The *cyt b* marker resolved species better than the *16S* marker, which had the highest number of species pairs with similar PCR-HRM profiles. While it is expected that longer PCR products would have more than one melt peak due to multiple melting domains (Wittwer, 2009), *cyt b* (~383 bp) and 16S rRNA (~200 bp) PCR products tended to have

simple single-peaks compared to *CO1* PCR products (~205 bp), for which many samples had multiple peaks, generating a greater diversity of unique HRM profiles.

4.3 HRM identification of covertly sampled meat from butcheries in Naivasha, Kenya

Out of the 90 meat samples covertly sampled with support of the KWS from butcheries in the Naivasha region of Kenya (0°43' 0.01" N 36° 26' 9.28" E, about 77 km from the capital Nairobi). The town is in the vicinity of wild animal conservancies and game parks. Results showed one of the samples (from Kambi Samaki area) to be a giraffe bushmeat by PCR-HRM and subsequent *CO1* barcode sequencing confirmation **(Figure 4.7)**. The remaining 89 samples consisted of 49 (54.4%) sheep, 29 (32.2%) cattle, eight (8.9%) goats, and two (2.2%) pigs, while one (1.1%) sample failed to amplify **(Figure 4.8)**. Out of the 17 random samples whose species identity were given by the butcher at the point of sale, six samples (35.3%), sold as goat meat, were confirmed by PCR-HRM analysis to be sheep meat.

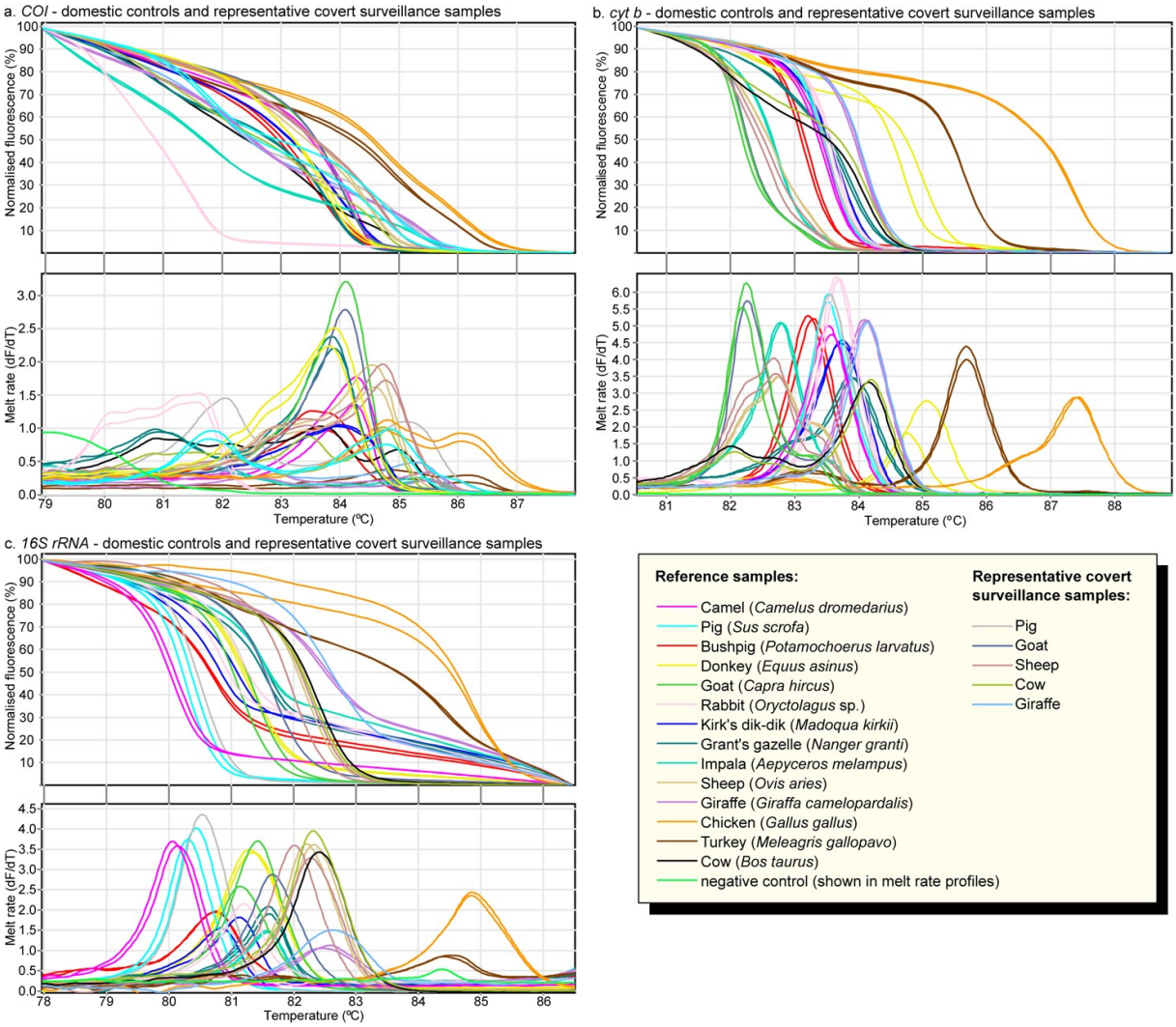

Figure 4.10: Distinct normalized HRM and melt rate profiles of domestic reference and representative covert surveillance samples. Normalized HRM profiles are represented as percent fluorescence and melt rates are represented as change in fluorescence units with increasing temperatures (dF/dT) for (A) COI (B) cyt b and (C) 16S rRNA markers.

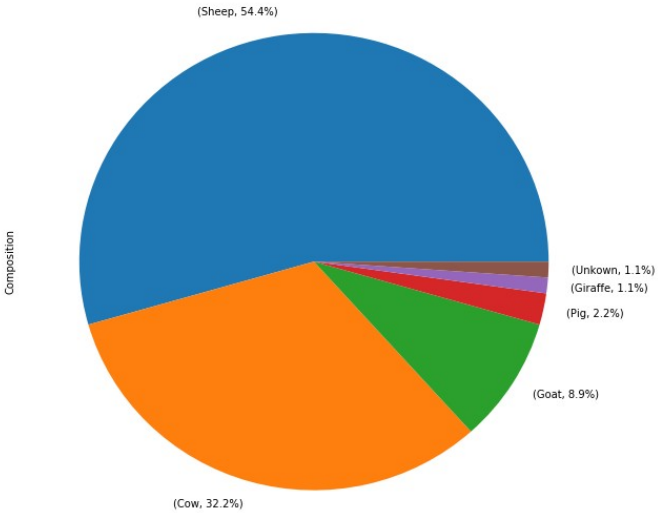

Figure 4.11: A pie chart summarizing the percentage species distribution of the covert samples from Naivasha as determined using PCR-HRM.

4.4 Covert samples sequence analysis

Out of the 90 covert samples eight samples were sequenced to ascertained slight profile variations observed in HRMA and to confirm species identified by HRMA. **Figure 4.9** shows the Geneious analysis results.

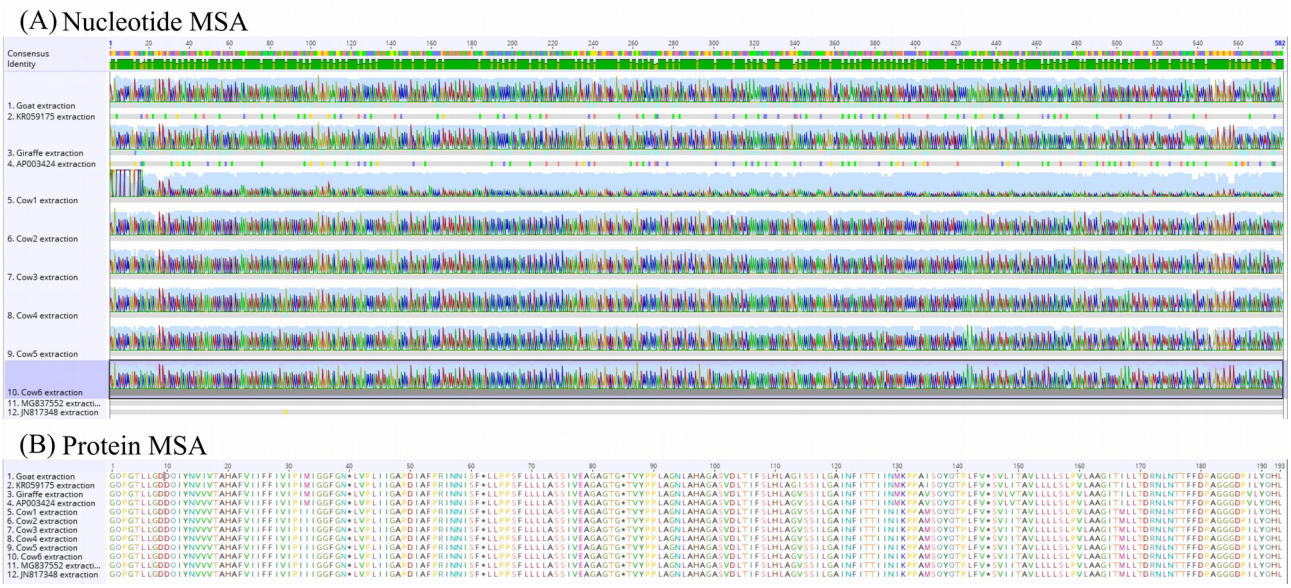

Figure 4.12: Multiple sequence alignment showing the alignment of the eight covert samples sequenced as representatives and GenBank references using MUSCLE for nucleotide (A) and protein (B).

CHAPTER FIVE

5.0 DISCUSSION

This study clearly demonstrates the utility of PCR-HRM analysis of three mtDNA markers for efficiently differentiating and identifying the vertebrate species origin of unknown tissue samples. Using PCR-HRM technique, it was possible to differentiate domestic and wild animal species native to East African region by HRM analysis of short *COI*, *cyt b*, and *16S* rRNA gene PCR amplicons and blindly identify giraffe (an illegal bushmeat) among the meat samples purchased from butcheries in Naivasha town, using forensic barcode sequencing only for confirmation purposes. The PCR-HRM approach therefore represents a valuable addition to molecular forensic pipelines for the surveillance of illegal bushmeat, as it eliminates the need for mass barcode sequencing of specimens, most of which tend to be legally traded domestic animal samples. These assays can also be effectively used for biodiversity surveys from the blood-meals of hematophagous invertebrates (Lee et al., 2015) and for consumer protection purposes to ensure that meat products for consumption are labeled properly.

The analysis of PCR-HRM profiles generated by the three DNA markers from 10 domestic and 24 wild vertebrate species endemic to East Africa, demonstrated the capacity of this approach to differentiate a large range of species. While all species were differentiated by considering the combined analysis of HRM profiles generated by the three DNA markers, some of these markers were not individually capable of distinguishing particular vertebrate species pairs, due to varied resolution strengths (Boonseub et al., 2009; Ficetola et al., 2010). Though some studies have only considered one marker (Peña et al., 2012) or two different DNA markers (Naue et al., 2014), this study demonstrates increased robustness of species

identification by PCR-HRM when using combined analysis of three DNA markers. While the reliability of sequencing, as widely applied in species identification, cannot be doubted, the high costs associated with it may not be sustainable in some instances, especially where large sample sizes are analysed (Villinger et al., 2017). This results support PCR-HRM as a quicker, cheaper, and relatively easy-to-work-with method than sequencing-based conventional methods (Erali, & Wittwer, 2010; Vossen et al., 2009). The use of short gene targets of 130 bp for species identification, that works best with HRM, have been shown to have similar performance to longer targets (Meusnier et al., 2008) and are more suitable for environmental samples.

By enabling rapid differentiation of commonly consumed domestic species from wild vertebrate species, PCR-HRM eliminates commonly sold and used domestic species samples from further analysis required to generate forensic evidence for prosecution. This allows efficient and sustainable monitoring of potential illegal bushmeat trade as a long-term activity in deterring illegal poaching of wildlife.

Analysis from this study demonstrate the applicability of PCR-HRM analysis to illegal bushmeat surveillance with a small-scale covert surveillance exercise conducted in collaboration with the KWS. Using the three marker PCR-HRM assays to screen 90 meat samples sold as domestic livestock meat in Naivasha, Kenya, giraffe was identified as meat sold illegally but disguised as domestic meat. This was surprising as we expected poaching of much smaller, easier-to-trap, ruminants to occur more frequently among illegal bushmeat (Stella et al., 2012). Poached giraffe meat products are of particular concern as giraffe

populations have been declining in the region. The latest update of the International Union of Conservation of Nature (*IUCN Red List2018-2)* only recently added two of the nine sub-species of giraffes to the "Critically Endangered" category. Five out of seven assessed giraffe sub-species are categorised between "Near Threatened" to "Critically Endangered" (GCF, 2018) (*https://giraffeconservation.org/2018/11/14/giraffe-subspecies-update/*). Further, the sale of illegal bushmeat as livestock meat also presents a public health concern as unsuspecting consumers may be exposed to heightened risk of contracting zoonotic diseases from bushmeat (Judson et al., 2016; Leroy et al., 2004). Additionally, sheep meat was sold from local butcheries as goat meat among the covertly sampled meat. This further demonstrates the potential utility of PCR-HRM for surveillance by consumer protection agencies, such as the Kenya Bureau of Standards (KEBS), which could, in turn, inform policy formulation and law enforcement.

During validation of several elephant reference samples, we identified two sets of distinct HRM profiles among the KWS stock samples of Kenyan savannah elephants. By *COI* barcode sequencing confirmation, we determined that some samples amplified mtDNA sequences associated with forest elephant populations, which are thought to be extinct in the region (Litoroh et al., 2012). This finding could represent an artefact of past hybridization (Groves, 2016) between female forest elephants and male savannah elephants, after which the forest elephant mtDNA persisted in East African savannah elephant populations. Therefore, our method can also be used to identify mtDNA variants within populations. Our findings suggest that further screening of savannah elephant samples by PCR-HRM could determine the frequency of forest elephant mtDNA in savannah elephant populations and potentially

vice versa. This could inform accurate documentation of the occurrence or non-occurrence of distinct elephant sub-species in the region, which may affect conservation efforts.

The ability to distinguish by PCR-HRM between diverse ungulates, including buffalo, cow, waterbuck, sheep, goat, and three different zebra species, suggests that the three-gene DNA marker PCR-HRM method can differentiate even closely related species. Despite the differences in tissue type and PCR cycling conditions, we noted that the *cyt b* and *16S rRNA* HRM profiles for cow, goat, sheep, pig, and chicken DNA samples obtained in this study are comparable to those previously obtained from mosquito blood-meals analyses to determine host feeding preferences (Ogola et al., 2015; Omondi et al., 2015). These observations support the overall reproducibility of the method.

The differences exhibited by the DNA markers in their ability to differentiate any two species by PCR-HRM, strongly support the complementarity in using the combination of the three DNA markers in analyses, to address marker-specific in differentiating certain vertebrate species. Previous studies also highlighted the importance of marker complementarity in screening mosquitoes for blood-meal sources using HRM (Omondi et al., 2015). Even though this means up to three PCR assays could be required to confidently identify some species, the overall time and cost is still cheaper, as the runs can be done simultaneously. Moreover, there was still no need for large-scale sequencing of all PCR amplicons.

Consistent with a previously identified marginal positive correlation between amplicon length and species resolution based on *COI* sequences and sequence amplicons of >200 bp

(Meusnier et al., 2008), we found that *16S rRNA* with an amplicon size of about 200 bp had the lowest resolving power compared to *COI* (~205 bp) and *cyt b* (~383 bp). The ~200 bp *16S* rRNA sequence might have been too short to incorporate sufficient sequence variations required to distinguish species as effectively as the other two markers. The observation that analysis of *COI* and *cyt b* HRM profiles discriminated vertebrate species better than *16S* rRNA HRM profiles is consistent with a study that found *16S* rRNA sequences to be 2.5 times less variable than *COI* and *cyt b* sequences in rodents within the Praomyini tribe (Nicolas et al., 2012). In contrast to previous studies that have investigated the use of HRM analysis to differentiate vertebrate species using different primers to target discrete taxonomic groups (Naue et al., 2014), our study used three different sets of universal primers to "globally" differentiate a large repertoire of species. This suggests that its applicability could be much broader than previously published assays.

From the double-blind validation analysis using covert samples from local butcheries, the power of three-gene DNA marker HRM analysis (HRMA) is evident. Out of the 90 samples drawn from the surveillance exercise, we only sequenced eight representative DNA samples with unique HRM profiles to confirm species identifications. This translates into 91% reduction in sequencing costs compared to direct sequencing of all the 90 samples. Among the eight samples sequenced, one was identified as giraffe by HRMA and only needed sequencing confirmation for depositing in nucleotide database and future forensic prosecution purposes. The other seven were representative sequences of samples with HRM profiles matching those of domestic livestock species. Despite the challenges of sampling during times not favoring the concealed nature of illegal bushmeat trade (from late morning to early evening) and

having to deal with mitigating the alerting-appearance of the KWS covert operations team and vehicle, we managed to find one bushmeat specimen among the samples collected. This shows that the problem of illegal bushmeat trade indeed exists in the sampled area.

5.2 CONCLUSIONS

1. Three-gene HRMA is capable of differentiating vertebrate species spanning a wide range, in some cases up to the subspecies level. While two genes *CO1* and cyt b would be sufficient for this purpose in most cases, 16S rRNA may also be used to increase robustness
2. Three-gene HRMA is sustainable for routine surveillance compared to conventional long barcode sequencing. This is especially so for high throughput assays dealing with large samples, as demonstrated in the covert exercise
3. The hunting and consumption of illegal bushmeat, including those ear-marked as endangered species, such as giraffe, is a problem that exist and must therefore be tackled
4. 16S rRNA has the lowest resolution for species targeted in the study, cyt b has better resolution than the former, while *CO1* has the best resolution, except with a poor amplification efficiency than the former two markers

5.3 RECOMMENDATIONS

1. Wildlife management organizations such as KWS to adopt HRMA for covert surveillance as a means to sustainably speed up their forensic pipeline for evidence processing in the prosecution of perpetrators by eliminating non-informative samples from further analysis
2. The development of HRMA profile databases for additional species and generate

consensus references covering divergent aspects such geography and extraction assay differences

3. HRMA to be adopted for consumer protection surveillance to counter the deception in butcheries
4. Frequent and broader covert surveillance to capture the real extent of the illegal bushmeat menace, which will further guide the development of policies against the vice.

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