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Development and Application of Molecular Tools for the analysis of Animal Parts and Derivatives

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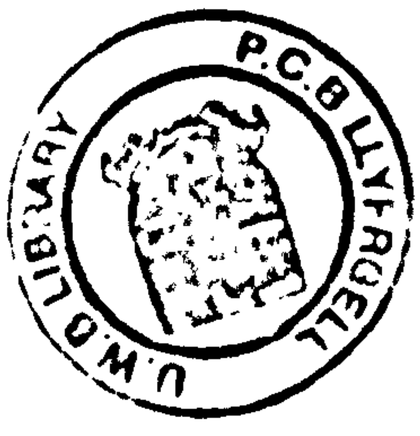

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In Loving Memory of Auntie Edna,
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Abstract

Illegal wildlife trade presents a serious threat to the long-term survival of certain species, which can have serious ecological and socio-economic consequences. Officials attempting to enforce wildlife trade legislation are often hampered by difficulties associated with the accurate identification of such items. In response, this thesis describes the development and application of molecular tools for the analysis of animal parts and derivatives, with reference to two main contemporary issues; namely, the illegal trade in bear (Ursid) parts and derivatives, and the illegal trade in rhinoceros parts and derivatives.

A qualitative lateral flow immunoassay (LFIA) dipstick was developed for bear serum albumin detection. The visual detection limit was 10 ppm of bear serum with a reaction time of 5 min. The LFIA was validated on serum, blood, skin and liquid bile, and was able to detect bear albumin in all these sample types. Items confiscated during enforcement activities were also tested and the results confirmed by DNA sequence analysis. PCR primers were designed such that DNA from ursine species could be amplified from samples containing mixed-species DNA. The LFIA accurately identified genuine bear bile crystals and bear bile capsules, although it was unable to consistently identify bear bone and some of the more complex traditional Asian medicines (TAM). The test can be performed by persons with little or no scientific training and may provide a novel method for customs and law enforcement officials to screen purported bear bile samples and gallbladders in the field.

The feasibility of using mitochondrial and nuclear DNA for individual identification in the white rhinoceros, *Ceratotherium simum*, was assessed. A molecular method for sexing rhinoceros samples was developed and incorporated into a short tandem repeat (STR) profiling assay, which was then partially-validated for forensic use and used to screen various rhinoceros sample types (horn, blood, tissue) from wild and captive white rhinoceros. The recovery of nuclear DNA (microsatellites) from rhinoceros horn is demonstrated, opening up the possibility of being able to match confiscated horn samples to a particular rhino carcass.

A preliminary assessment of the authenticity of exotic meat products sold in the UK was performed, and 40% of items were found to be fraudulent. The implications of this finding are discussed, and the study highlights some of the difficulties associated with mitochondrial DNA-based species identification.

Preface

This thesis details the research conducted as part of my Ph.D. study at the School of Biological Sciences, College of Natural Sciences, Bangor University (UK). It serves to document the work completed during the course of my studies, completed during the period December 2005 – June 2009 and partly funded by Objective One – European Social Fund and Wildlife DNA Services (WDNAS).

This research documented here was conducted in response to the growing problem of illegal wildlife trade. Such trade threatens the long-term survival of certain species, which in turn can have serious ecological and socio-economic consequences. Attempts to enforce trade legislation are often hampered by difficulties associated with the accurate identification of wildlife products, which are generally highly processed and lack diagnostic morphological characteristics. In this thesis, we are particularly concerned with the trade in animal parts and derivatives.

The thesis contains five chapters. In the first chapter, the problem of illegal wildlife trade and the difficulties of legislation enforcement are explored and discussed in detail, followed by an overview of the techniques currently applied to the analysis of animal parts and derivatives. The field of wildlife forensics is introduced, along with a brief discussion of the importance of validation during technique development and application in this area of research. In chapter 2, the illegal trade in bear parts is discussed and the primary barriers to the enforcement of the relevant legislation are identified. In response, immunological and genetic approaches are developed and applied to the analysis of bear parts and derivatives, and the techniques validated for forensic use where possible. To assist the broader application of this work to the field of wildlife forensics the work in this chapter has been published (see Appendices 3 and 4).

In the third chapter, the illegal trade in rhinoceros parts and derivatives is considered, with particular attention to poaching and illegal trade in the southern white rhinoceros. The history of the trade and contemporary issues are discussed, and genetic assays (nuclear and mitochondrial) for the analysis of rhinoceros samples are developed. To assist the broader application of this work to the field of wildlife forensics the work in this chapter has been partially published (see Appendix 5), with a view to publishing more of this work in the future.

The fourth chapter details a preliminary study looking at the authenticity of exotic meat products sold in the UK, with discussion of the implications of the findings in a broader context. Finally, in chapter 5, the main findings of the research reported here and their implications are discussed in the light of the present status of the field of wildlife forensics and current legislation, and key areas for future work identified.

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Abbreviations

AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic local alignment search tools
BOLD	Barcode of Life Data Systems
BSA	Bovine serum albumin
BSE	Bovine Spongiform Encephalopathy
BU	Bangor University
CBOL	Consortium for the Barcode of Life
CE	Capillary electrophoresis
CE-SDS	Sodium dodecyl sulphate polymer-filled capillary gel electrophoresis
CITES	Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora
CNBr	Cyanogen bromide
COI	Cytochrome oxidase subunit I
COTES	Control of Trade in Endangered Species
DAB	DNA Advisory Board
Defra	Department for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
DTB	Di-sodium tetra borate
ELISA	Enzyme-linked immunosorbent assay
FTIRA	Fourier Transform Infrared Analysis
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IEF	Iso-electric focusing
IgG	Immunoglobulin
ISFH	International Society for Forensic Haemogenetics
IUCN	International Union for Conservation of Nature
IZW	Leibniz Institute for Zoo and Wildlife Research
JNCC	Joint Nature Conservation Committee
LC	Liquid chromatography
LFIA	Lateral flow immunoassay
LGC	Laboratory of the Government Chemist
ML	Maximum likelihood
MS	Mass spectrometry
mtDNA	Mitochondrial DNA
MudPIT	Multidimensional protein identification technology
MVP	Minimum Viable Population
NCBI	National Centre for Biotechnology Information
nDNA	Nuclear DNA
NGO	Non-governmental Organisation
NMR	Nuclear magnetic resonance
NWCU	National Wildlife Crime Unit
NWFP	Non-wood forest products
PAGE	Polyacrylamide gel
PAW	Partnership Against Wildlife Crime
PCR	Polymerase chain reaction
pI	Isoelectric point
POC	Point of Care
pp	Posterior probabilities
PSA	Pig serum albumin
PVA	Polyvinyl alcohol
QA	Quality assurance

QC	Quality control
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassays
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SEM	Scanning Electron Microscopy
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
STR	Short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
TAM	Traditional Asian Medicine
TLC	Thin layer chromatography
UDCA	Ursodeoxycholic acid
UP	University of Pretoria
VNTR	Variable Number of Tandem Repeats
WDNAS	Wildlife DNA Services
WSPA	World Society for the Protection of Animals
WWS	Working Wash Solution

Published Work

Peppin L, McEwing R, Carvalho GR and Ogden R (2008) A DNA-Based approach for the forensic identification of Asiatic black bear (*Ursus thibetanus*) in a traditional Asian medicine. *Journal of Forensic Sciences*, 53(6):1358-1362 (Appendix 3)

Peppin L, McEwing R, Webster S, Rogers A, Nicholls D and Ogden R (2008) Development of a field test for the detection of illegal bear products. *Endangered Species Research*, Special Edition: Forensic Methods in Conservation Research (Appendix 4)

Peppin L, McEwing R, Ogden R, Hermes R, Harper C, Guthrie A and Carvalho GR (2009) Molecular sexing of African rhinoceros. *Conservation Genetics*, Available online April 2009 (Appendix 5)

Chapter 1

General Introduction

Chapter 1:
General Introduction

1.1 Wildlife Trade

The term 'wildlife trade' refers to the sale or exchange of any wild animal or plant resources, whether they be whole, living specimens or highly processed parts and derivatives. Such resources rank amongst the oldest commodities to be traded on the international market (Iqbal 1993; Forsyth 2005; Dubey 2007), and despite advances in agriculture, materials science and the development of sophisticated synthetic medicines, wildlife and wildlife products are still in high demand today.

The modern wildlife trade is highly diverse, involving thousands of species made into countless different products. The species concerned are traded for a wide variety of reasons, such as for food, medicine, horticulture, companion animals, clothing, fuel, ornamental and cultural purposes (TRAFFIC 2008). The trade occurs at local, national and international levels, ranging in scale from small, local income generation to large commercial operations such as the timber and fisheries industries. Given these complexities, it is hardly surprising that any attempt to describe the wildlife trade must begin by acknowledging that this cannot be done with any real accuracy (Roe *et al* 2002).

Legal international wildlife trade is estimated to be worth around EUR 249 billion per year, with timber and fisheries accounting for 90% of this value (Engler & Parry-Jones 2007). Table 1.1 details estimates of the annual global value of some of the main wildlife commodities found on the market. These conservative figures are based on declared import values, and do not include trade occurring at local and national levels. Furthermore, wildlife trade is poorly documented in terms of the species and the number of individual plants and animals involved, and major discrepancies have been identified in the published statistics (e.g. Blundell & Mascia 2005; Clarke *et al* 2006). Consequently this table is far from a complete representation of the value of wildlife trade either globally or in the EU, however it serves as an indication of its scale and economic importance.

Although the exact scale of the wildlife trade is unknown, wildlife products undoubtedly play a significant role in the welfare of human populations around the world, especially in developing countries. Trade in wild species is a major component

Commodity	Estimated Global Value (EUR)
Live Animals	
Primates	75 million
Cage Birds	38 million
Birds of Prey	5 million
Reptiles, including Snakes & Turtles	31 million
Ornamental Fish	257 million
Animal Products for Clothing/Ornamental Items	
Mammal Furs & Fur Products	4 billion
Reptile Skins	255 million
Ornamental Corals and Shells	85 million
Natural Pearls	57 million
Animal Products for Food (Excluding Fish)	
Game Meat	365 million
Frogs Legs	40 million
Edible Snails	60 million
Plant Products	
Medicinal Plants	1 billion
Ornamental Plants	11 billion
Non-Wood Forest Products (NWFPs)	9.5 billion
SUBTOTAL (excl fisheries products & timber)	**26.7 billion**
Fisheries Food Products (excl. aquaculture)	68.6 billion
Timber	154 billion
TOTAL	**249 billion**

Table 1.1 Estimates of global wildlife trade values in 2005. Reproduced from Engler & Parry-Jones (2007). NWFPs include foods (nuts, fruits, mushrooms, honey, game, gums), food additives (spices, herbs, flavorings, sweeteners), fodder, fibres (furniture, clothing, construction), fragrances for perfumes ornamental pods and seeds, resins, oils, plant and animal products of medicinal value.

of rural incomes in such areas, and can form the basis of local economies (Roe *et al* 2002). The subsistence use of wild animals for food represents an important source of protein, contributing at least a fifth of the animal protein in rural diets in over 60 countries (Bennett & Robinson 2000), and reliance on wild meat is growing in some areas due to increasing human populations and poverty (TRAFFIC 2009). Trees and other plants provide fuel and construction materials, and around 80% of people in the developing world rely on non-wood forest products (NWFPs) for some purpose in their everyday lives (FAO 1999). A similar proportion of the world's population rely on traditional medicines – most of which are plant and animal based – for their primary healthcare needs (WHO/IUCN/WWF 1993). Wildlife also represents a sizeable contribution to the economies of developed countries – the estimated declared import value of wildlife products in the EU alone was approximately EUR93 billion in 2005 (Engler & Parry-Jones 2007).

1.2 Trade in Animal Parts & Derivatives

This thesis is specifically concerned with the trade in animal parts and derivatives which, although a small fraction of overall wildlife trade, is still a multi-billion dollar business (see Table 1.1), a proportion of which is illegal. The trade in animal parts and derivatives is driven by a number of social and economic factors (TRAFFIC 2008). In developing countries, growing human populations increase demands for food, clothing, medicine and income generation. In the developed world, increases in tourism and consumer demands for souvenirs, fashion trends, interest in alternative and traditional medicines, and growing expatriate populations which demand wildlife products be shipped over from their home countries. Modern transportation methods and, increasingly, the internet, facilitate delivery of wildlife and wildlife products throughout the world to these consumer markets, and increasing wealth in some areas has made luxury items (once off-limits to many people because of their high cost) affordable.

Given the scale of the wildlife trade it is not surprising that detrimental effects have been observed in a number of species, the most direct and obvious of which is a reduction in population sizes in the wild. Economic theory predicts that exploitation of a species should cease before ecological extinction occurs; as populations decrease, the costs associated with exploitation spiral and utilisation of the species should become unprofitable (Clark 1990). However, for many species, increasing rarity makes them systematically more valuable (Courchamp *et al* 2006). For example, rare

species are more desired by collectors and command higher market value (e.g. butterflies [Slone *et al* 1997]). Furthermore, rare specimens used in traditional medicine are perceived to have stronger medicinal powers and are highly sought after by consumers (e.g. swim bladders from the Chinese bahaba, Sadovy & Cheung 2003). When the value of a specimen is closely linked to its scarcity, a vicious circle can ensue whereby as a species becomes more endangered, so its value increases, as do the financial rewards for those individuals able to supply the demand (Cook *et al* 2002; Hayman & Brack 2002). The species becomes increasingly targeted despite declining populations making it increasingly difficult to locate individuals. Therefore contrary to economic theory, an 'anthropogenic Allee effect' is observed whereby ever increasing demand for specimens leads species into an extinction vortex (Courchamp *et al* 2006).

Indeed, the trade in animal parts has significantly reduced wild populations of certain species, which can have far-reaching implications. An example of this is fruit bat populations in the South Pacific. Fruit bats have been a delicacy for indigenous people on the island of Guam for millennia, but when traditional hunting methods were replaced with more efficient fire arms, local bat populations were virtually wiped out. Other Pacific islands began exporting bat carcasses to Guam (Wiles 1992; Hemley 1994), in a trade that was lucrative but which reduced bat populations throughout the entire region. Fruit bats play important ecological roles as pollinators, and declining populations will have a negative impact on the plants dependent upon them for propagation (Cox *et al* 1991). Declines in these plants will in turn affect other species, demonstrating how the removal of one key species can set in motion a chain of events which leads to severely impaired ecosystems and a collapse of biodiversity in the region (Marshall 1983; Cox *et al* 1991).

Fruit bats are not an isolated example. Many animal species affected by trade play important ecological roles. Elephants and rhinos (killed for their ivory and horn, respectively) shape the environment by knocking down trees and small bushes, thus maintaining the grasslands that humans use to graze cattle, and play important roles in nutrient cycling and seed distribution (Naylor 2004). Large predators such as wolves, cougars (killed for their skins) and bears (killed for trophies and for use in traditional medicine) keep populations of prey species in check and are key links in food chains (Leopold *et al* 1947; Berger *et al* 2001; Ripple & Beschta 2006). Corals (taken for ornamental use) build reefs which house a great diversity of marine life, provide nursery grounds for many commercially valuable marine species, and serve

as natural barriers against beach and island erosion (Miththapala 2008). The removal of such species can potentially result in a cascade of negative effects throughout the rest of the ecosystem, which will in turn impact upon human health, welfare and prosperity. It is therefore important to ensure that wildlife trade does not threaten species survival (Hemley 1994), and that an appropriate detection and enforcement framework is in place.

1.3 Regulation of Wildlife Trade

The Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) was established in response to the realisation that wildlife trade was seriously threatening the long-term survival of certain species. The treaty, which came into force in 1975, has 175 member parties (CITES 2009) and is the largest and arguably most effective international wildlife conservation agreement in the world (Ong 1998). It decides when international trade in a species can continue unchecked, when it must slow, or stop altogether (Hemley 1994). Although the primary aim of the Convention is to ensure that international trade in specimens of wild animals and plants does not threaten species survival (CITES 2009), the treaty also attempts to balance the interests of preserving biodiversity with the interests of developing nations in using their natural resources to further their economic development.

The convention is centred upon three appendices, which list around 5,000 animal and 28,000 plant species. Contrary to popular belief, not all endangered species are listed within CITES, and not all CITES-listed species are endangered. Rather, CITES lists those species recognised as threatened by or potentially threatened by international trade. For example, despite both being listed as 'endangered' on the IUCN Red List of Endangered Species, the African wild dog (*Lycaon pictus*) and the Ethiopian wolf (*Canis simensis*) are not listed in CITES, as there is no commercial trade in these species. However other members of the family Canidae such as the grey wolf (*Canis lupus*) are relatively common, yet are listed within CITES due to the large volumes of trade in their pelts (Ginsberg & MacDonald 1990). Furthermore, a species can only be listed on CITES appendices if adequate data on exploitation levels exists. The absence of a species from the appendices could therefore be due to a lack of information, rather than a lack of trade.

The different CITES appendices afford various levels of protection, which reflect the status of the species in the wild (see Table 1.2). The convention applies in situations

Appendix	Description	Trade Permitted
I	Includes all species threatened with extinction which are or may be affected by trade	Strictly regulated; trade only authorised in exceptional circumstances.
II	Includes: (a) all species which although not necessarily now threatened with extinction may become so, unless trade in specimens of such species is subject to strict regulation in order to avoid utilisation incompatible with their survival (b) other species which must be subject to regulation in order that trade in specimens of certain Appendix II species may be brought under control, e.g. 'look-alike' species (c) specimens of an animal species included in Appendix I bred in captivity for commercial purposes, or of a plant species included in Appendix I artificially propagated for commercial purposes, shall be deemed to be specimens of species included in Appendix II	Regulated
III	Includes all species which any party identifies as being subject to regulation within its jurisdiction for the purpose of preventing or restricting exploitation, and as needing the co-operation of other Parties in the control of trade	Regulated

Table 1.2 Description of the three CITES Appendices. Based on the text of the convention, available at www.cites.org

where species listed within its appendices are moved across international boundaries. While a specimen remains within a country, CITES is irrelevant and trade or movement is regulated by national legislation. The movement of CITES species across an international border must be authorised by permits issued by the countries involved. Import and export permits are only issued if the authorities concerned are convinced that the trade will be non-detrimental to the species in the wild. However, the difference between non-detrimental and sustainable trade and what determines a threat at both the national and international levels has been extensively debated but never resolved (Ginsberg 2002).

Each member country designates one or more management authorities to be in charge of administering CITES, and one or more scientific authorities to advise them on the effects of trade on species' status. For example, Defra and its executive agency – Animal Health – are the management authorities responsible for ensuring correct CITES implementation in the UK. The UK also has two independent Scientific Authorities; the Joint Nature Conservation Committee (JNCC) for animals, and the Royal Botanic Gardens, Kew for plants.

Appendix I permits are very strictly controlled and authorised only in exceptional circumstances, when the movement of the specimen is primarily for non-commercial purposes (for example, scientific research). Movement of Appendix I species requires both import and export permits (or re-export certificate, if applicable), issued by the management authorities of the relevant countries.

Appendix II contains species that are not currently threatened with extinction, but that may become so if trade is not controlled. It also lists 'look-alike' species, that is, species of which specimens in trade look the same as those listed for conservation purposes, the logic being that it is impossible to regulate trade in one if it cannot be distinguished from the other. International trade in Appendix II species requires the issue of an export permit (or re-export certificate). Unlike trade in Appendix I species, no import permit is required under CITES rules, although in some countries – which have applied stricter measures than required by CITES – a permit is necessary.

Species in Appendix III are subject to the lowest levels of regulation and are included at the request of a party that already regulates trade in the species but needs the co-operation of other countries to prevent unsustainable or illegal exploitation.

International trade in Appendix III species requires an export permit (or re-export certificate) from the relevant management authority.

Some countries enforce stricter regulation than is required by CITES. For example in the EU, trade in wildlife is regulated though the European Union Wildlife Trade Regulation. The Regulation enforces CITES within the EU and provides additional measures for the conservation of traded species. Under the Regulation, species are classified into a series of four Annexes (A-D) which go beyond CITES in a number of respects (see Table 1.3). For example, Annex A, which affords the highest level of regulation, contains all of CITES Appendix I species. However it also contains some CITES Appendix II and Appendix III species, and even some non-CITES species. In the UK, the Control of Trade in Endangered Species (COTES) Enforcement Regulations create the offences and penalties for breaches of the EU Wildlife Trade Regulation, and set out the powers available to police to investigate such offences.

1.4 Difficulties Associated With CITES Enforcement

When a species is listed in an appendix, all parts and derivatives of that species are also included unless otherwise stated. Indeed, much CITES-regulated trade relates not to live specimens but to animal-derived products, both raw and processed, that are 'readily recognisable' (CITES Article 1b). 'Readily recognisable' is interpreted as any specimen which appears from an accompanying document, packaging, label, or any other factor to be a part or derivative of a listed species. However animal products are often highly processed and/or lack diagnostic morphological characteristics, making species identification difficult (see Fig 1.1a-i). In some countries, for example the UK, a policy has been adopted whereby a product label claiming the presence of a CITES-regulated species provides sufficient grounds for prosecution. However this policy has not been universally adopted by all countries party to CITES, and in other areas it is necessary to demonstrate the presence of the species in the product before prosecution can occur. This is because items labelled as being or containing protected species may not be genuine, and there is a high incidence of fake and fraudulent items, particularly of high-value wildlife commodities (Espinoza *et al* 1993; Nowell & Jackson 1996; Roman & Bowen 2000). Furthermore, manufactured items containing protected species (traditional Asian medicines, TAMs) are often unlabelled (Govind & Ho 2001; Peppin *et al* 2008), making them easy to conceal, transport and sell illegally. The situation is complicated by the fact that in some circumstances, certain populations of a species are afforded higher levels of protection than others. For example, musk deer (*Moschus* spp.), whose parts are

Annex	Description
A	- All CITES Appendix I species - Some CITES Appendix II and III species, for which the EU has adopted stricter domestic measures - Some non-CITES species
B	- All other CITES Appendix II species - Some CITES Appendix III species - Some non-CITES species
C	- All other CITES Appendix III species
D	- Some CITES Appendix III species for which the EU holds a reservation - Some non-CITES species

Table 1.3 Annexes of the European Union Wildlife Trade Regulation.

Figure 1.1 Examples of wildlife products found in trade. (a) sea cucumbers[1] for sale in a TAM store (b) turtle soup[2] (c) elephant ivory ornament[3] (d) biltong[4] (e) crocodile skin handbag[5] (f) Shatoosh shawl[6] (g) Russian and Iranian caviar[7]

Images from http://commons.wikimedia.org
Authors: [1]Chris 73, [2]Chensiyuan, [3]Jastrow, [4]Liftarn, [5]Arpingstone, [6]US Fish and Wildlife Service (Walton LaVonda), [7]The Ogre

highly prized in TAM, are classified as Appendix I or II depending on their geographic origin. Populations in Afghanistan, Bhutan, India, Myanmar, Nepal and Pakistan are listed in Appendix I; all other populations are listed in Appendix II. Similarly, captive-bred individuals can have different listings to their wild counterparts; Appendix I specimens bred in captivity for commercial purposes are treated as Appendix II specimens, thus permitting them to enter trade. These 'split-listings' between appendices present obvious management problems, and contribute to the complexities of enforcement (Jenkins 2000). Analysis is complicated even further when hybrids are considered. CITES states that when hybrid animals have in their recent lineage (whereby 'recent lineage' refers to the previous four generations) one or more species included in Appendix I or II, the hybrid is treated as if it were a specimen of that appendix, regardless of whether it's other ancestors are CITES-listed or not.

Furthermore, specimens acquired before 1st June 1947 are exempt from CITES regulation (Article VII paragraph 2). Specimens acquired before 1st June 1947 which were subsequently re-worked *are* subject to CITES restriction. Enforcement officers therefore not only need to determine what species an item is, but also how old it is, or at what point any re-working of the item occurred.

1.5 Illegal Wildlife Trade

The high demand for certain wildlife commodities, and the rich financial rewards for those individuals who can supply this demand, inevitably leads to illegal trade. Ironically, CITES-listed and other species protected by law command higher prices than those not protected, so whilst a CITES-listing confers greater trade controls for a species, it also highlights the species as 'rare' which can inadvertently increase the market value. For example, shahtoosh shawls, made from the hair of CITES-listed Tibetan antelope, can retail for around £6,500, whereas pashminas, a similar product made from the hair of non-CITES listed domestic goats, retail for as little as $30 (Donn & Yates 2002).

It is often stated that wildlife constitutes the third largest illegal traffic after drugs and arms, however the data are not available to validate this statement (Pete Younger, Interpol Wildlife Crime Officer, *pers.comm.*). Attempts have been made by several organisations, including Interpol and WWF, to define the scale of illegal wildlife trade, but due to the many inherent difficulties with the task the resulting estimates range widely from 25% to 70% of the legal trade (NWCU 2009). Often it is organisations

involved in legitimate trade – with inside knowledge and established contacts – that are the instigators of illegal trade, which makes detection even more difficult. For example, in 2001 US Caviar & Caviar was found guilty of a 5-year smuggling operation that resulted in the illegal import of caviar with a market value of approximately US $7.5 million dollars (Cook *et al* 2002). Wildlife trafficking is associated with lower risks of detection, prosecution and lower penalties than other forms of illegal trade (Hayman & Brack 2002). Limited resources, poor infrastructure, lack of expertise and low political will often limit enforcement capabilities and the scale of penalties applied. The combination of low risk and high reward has attracted the attention of organised crime elements that are becoming engaged in the more lucrative areas of trade (Cook *et al* 2002) and wildlife trafficking is increasingly coupled with drugs or arms trafficking, and even bank-rolling conflict (Brack & Hayman 2006).

A devastating consequence of illegal wildlife trade is the associated loss of human life. In parts of Africa, reports of park rangers and game wardens being killed in the line of duty are common (e.g. Duffy 2000; Moracco *et al* 2000; IRF 2009). Between 1996 and 2004, over 100 of the 700 park rangers in the Virunga National Park, had been killed (BBC 2004). Anti-poaching policies also lead to the deaths of the individuals perpetrating the crimes; during the first decade of Operation Stronghold, which had a "shoot first" policy at it's core, more than 178 suspected poachers were killed in Zimbabwe (Kelso 1993).

Wildlife trade is also a major source of disease, and an estimated 75% of emerging infectious diseases are zoonotic (Taylor *et al* 2001). Zoonotic diseases are far more frequent, widespread and contemporary than previously thought (Chomel *et al* 2007), and the emergence of these diseases is in part due to increased consumption of bushmeat across the world, especially in Central Africa and the Amazon Basin, where 1-3.4 million tonnes and 67-164 thousand tonnes respectively, are consumed each year (Karesh *et al* 2005). The urban demand for bushmeat combined with increasingly easy access to primate habitats provided by logging roads has increased the amount of hunting in Africa, which has in turn increased the frequency of human exposures to primate retroviruses and other disease-causing agents (Peterson 2003). For example, the consumption of nonhuman primates has been linked to T-lymphotropic virus (Wolfe *et al* 2004), outbreaks of Ebola (Georges-Courbot *et al* 1997) and the origin of human immunodeficiency virus (HIV) (Feng *et al* 1999). In Asia, international trade in small carnivores and bats is believed to have

been a significant factor in the emergence of severe acute respiratory syndrome (SARS) in 2003 (Bell *et al* 2004; Lau *et al* 2005). Global wildlife trade provides disease transmission mechanisms that not only cause human disease outbreaks, but also threaten livestock, international trade, rural livelihoods, native wildlife populations and the health of ecosystems (Karesh *et al* 2005). In addition, the financial cost of such disease outbreaks is considerable. Since the mid 1990s, disease outbreaks associated with wildlife trade have cost the world's economies over $80 billion (Newcomb 2004).

Unfortunately, not all parties are able to dedicate sufficient resources to implement CITES effectively. In such countries, the officials responsible for enforcement are often underpaid, and the profits that can be made trafficking contraband wildlife can be more than double the average annual salary (Zimmerman 2003). In such situations, criminal organisations often have more resources available to them than many governments and are able to pass on some of their profits to officials in the form of bribes (Zimmerman 2003). Such criminal operations threaten legitimate business. In Russia for example, illegal fishing is damaging legitimate fisheries by removing billions of dollars worth of fish from the Bering Sea (Mastny & French 2002). By obstructing the operations of legal businesses, organised criminal rings impede the growth of free markets in developing countries (Lee 1999) and the use of bribery tactics corrupts governments, undermines legitimate state structure, and threatens national stability (Guymon 2000), thus preventing the effective functioning of legal systems (Lee 1999). Corrupt governments deter foreign investment, thereby threatening that state's economic future (Zimmerman 2003). Furthermore criminals are able to channel the extensive profits generated from illegal wildlife trade into other illegal activities such as human and drug trafficking (Guymon 2000), causing further social and economic damage.

Governments are increasingly recognising the importance of wildlife legislation and of endangered species, both in terms of their economic roles (through sustainable trade and ecotourism) and their ecological importance. Growing connections with other forms of illegal international trafficking and organised criminal elements is providing further incentive to crack down on illegal wildlife trade. In some areas there has been a move towards strengthening collaboration, on national and international levels, between a range of bodies interested in law enforcement, species protection and animal welfare. Parties are recommended to improve enforcement by encouraging local communities to become involved in the management of their wildlife, by making

use of non-governmental expertise and the development of specialist teams for wildlife law enforcement. In the UK, such collaborative action is well established and joint meetings for government, police, customs and NGOs on the subject of wildlife law enforcement (including CITES) are held annually. A seminar is organised by a government-sponsored coalition of such bodies known as the Partnership Against Wildlife Crime (PAW) which is also active in the improvement of the quality of wildlife law enforcement, and has working groups on specialist topics such as forensics, training and access to information. Given that CITES, wildlife protection, animal welfare, animal health, customs and other offences are often interrelated, and an illegal incident may involve a number of these areas of law, the UK government has also established a National Wildlife Crime Unit which specialises in all aspects of wildlife law enforcement.

1.6 Wildlife Forensics

Put simply, 'forensic science' is the application of science to the law. In criminal investigations, scientific techniques are employed to link a suspect to a crime scene or to ascertain a sequence of events through the analysis of evidence samples. Due to the growing body of legislation governing the conservation, welfare and global movement of animals, quite often these samples are of animal origin. As well as being employed during investigations into crimes against wildlife, wildlife forensic techniques can also provide useful evidence in human murder and crime investigations (Allendorf & Luikart 2007). Biological material is easily transferred from animals to humans; for example, pet hairs easily stick to clothing and can be used to link a suspect to a crime scene. In this respect, much work has been dedicated to developing forensic profiling systems for companion animals such as dogs (Halverson & Basten 2005) and cats (Butler *et al* 2002), in order that animal samples recovered from a person or a place can be linked back to animal or origin.

In terms of the trade in animal products, the key questions that need to be addressed when determining whether or not the item contravenes trade regulations could be listed as:

(a) What species is it?
(b) What population / geographical location did it come from?
(c) Which individual did it come from?
(d) How old is it?

Not all questions need to be answered in each case – the type of analysis very much depends on the sample and the species in question. A range of molecular and chemical techniques have been applied to the analysis of animal parts and derivatives to try and answer some of these questions. As this thesis is concerned with the development of molecular techniques, chemical approaches are not considered in great detail here.

1.7 Species Identification Techniques

Species identification can only occur if species have been previously defined. More literature has been published on species concepts than perhaps any other subject in evolutionary biology (Sites & Marshall 2003). In excess of ten methods exist for delimiting species, with no single method being universally accepted. Such diverse terminology can cause problems for systematics which, in turn, can result in erroneous species assignments. Several studies demonstrate that the same scientific name can refer to two highly divergent molecular groups (e.g. Ludt *et al* 2004). Similarly, two valid species can be genetically very similar (e.g. Talbot & Shields 1996a,b). For complex organisms, the biological (BSC) and phylogenetic (PSC) species concepts currently dominate discussions (Isaac *et al* 2005), and legislation regarding wildlife tends to avoid defining 'species' in any way which would take sides in the scientific debate (Garnett & Christidis 2007). For example, CITES states that under the convention, "'species' and 'subspecies' refer to the biological concept of a species and do not require any further definition,' but also states that 'species' can relate to a 'geographically separate population' (CITES 2009).

Traditionally, morphological characteristics provided the principal source of evidence for defining species and organising them into groups during the construction of classifications. For some animal products, such as taxidermy specimens, whole animal carcasses or unprocessed animal skins, species identification may be performed using morphological approaches. Certain processed animal parts, such as fur and feather products, can also be identified using morphological techniques such as light microscopy or scanning electron microscopy (SEM) (Chandler 1916; Dove 1997; Donn & Yates 2002; Skeans 2003a,b). Even genuine ivory can be distinguished from imitation materials through examination of the pattern found on a cross section of the specimen (Shepherd 2002). However many animal products are processed to the extent that all diagnostic morphological characteristics have been lost. In response to this problem, a range of techniques have been developed for the

species identification of animal products, some of which are discussed here in more detail.

1.7.1 Immunological and Protein-Based Techniques

Many of the principal ideas of modern molecular biology were first published in the 1960s. Since then, the compositions of various biomolecules have been studied in great detail. Protein sequences were among the first to become available (Sanger & Tuppy 1951a,b), and, once homologous proteins were identified in different organisms, a molecular approach to species classification was quickly adopted. Half a century later, protein analysis still forms the basis of many methods of species identification, the three main approaches being (a) electrophoretic, (b) chromatographic and (c) immunological.

1.7.1 Electrophoretic Methods

Electrophoretic methods are based on separation of soluble proteins in an electric field. The separation can be conducted on polyacrylamide gels (PAGE), polyacrylamide gels containing denaturing agents (e.g. sodium dodecyl sulphate) (SDS-PAGE), hydrolysed starch, or isoelectric focusing (IEF) using agar or polyacrylamide gels (PAGIF).

Isoelectric Focusing (IEF)

Homologous proteins can vary between species in their isoelectric points (pI), thereby forming the basis of separation by IEF (Andrews 1986). Proteins carry positive, negative or zero charge depending on their local pH. The pH at which a protein has zero net charge is its pI. When a protein is placed in a pH gradient and subject to an electric field, it migrates towards the electrode with the opposite charge. At the point in the pH gradient which is its pI, it will be uncharged and will stop migrating. If the protein diffuses to a region outside of its pI, it will pick up charge and hence move back to the neutral position. In this way the proteins are 'focused' into sharp, stationary bands. Differences of only a few hundredths of a pH unit in isoelectric points are sufficient to resolve proteins from one another (e.g. Šťastná & Šlais 2005), so the technique is able to distinguish between homologous proteins from closely related species (Tinbergen & Olsman 1976). It can even distinguish between the same protein taken from different parts of an individual animal's body (Zechel & Weber 1978).

IEF is fast, sensitive, economical, requires no sophisticated equipment, and works on a clear, one-dimensional separation principle. Differences in experimental parameters (i.e. variation in methodology used between different laboratories and/or researchers) have been shown to have little effect on the results (Rehbein *et al* 1995). Furthermore IEF does not denature the proteins, thus any kind of subsequent investigations such as activity staining (i.e. to identify separated enzymes) or antibody detection is not hindered.

The technique has been applied to species identification of a range of fisheries products (e.g. Wei *et al* 1989; Rehbein 1990; Chen *et al* 1996; Smith & Benson 2001; Renon *et al* 2005) and to assist analysis the IEF protein banding patterns for many fish species are available from the US Food and Drug Administration (http://www.fda.gov). IEF has also been successfully applied to the species identification of meat (Montowska & Pospiech 2007), hair (Carracedo *et al* 1985; Carracedo *et al* 1987), and blood (Lawton & Sutton 1982). However it is not suitable for the analysis of mixed samples (Hsieh 2006), and is generally not suitable for the analysis of processed (e.g. heat treated or marinated) items, as most soluble proteins degrade rapidly under such conditions. Urea or non-ionic detergents may be added (e.g. urea-IEF) to aid analysis of denatured proteins, such as those extracted from cooked meats (Krzynowek & Wiggin 1979; Mackie 1980), but the pherograms obtained tend to be difficult to interpret and reproducibility is questionable.

SDS-PAGE and 2D Electrophoresis

2D gel electrophoresis separates proteins based on two properties. The first stage is an isoelectric focusing step (as described above) where proteins are separated according to their pI. The proteins are then treated with detergent (sodium dodecyl sulphate, SDS) and separated by polyacrylamide gel electrophoresis (PAGE). This separates proteins on the basis of their size to charge ratio, and occurs at right angles to the IEF. SDS-PAGE can be carried out as an independent technique, and not necessarily as part of 2D gel electrophoresis. For example, it has been applied successfully to the species identification of raw, cooked, smoked and brine cured food products (Zerifi *et al* 1991; Martinez *et al* 2001). However 2D gel electrophoresis has much greater resolving power (Issaq & Veenstra 2008).

Despite its high resolving power 2D gel electrophoresis has significant limitations. For example, it is quite a long and laborious process, and is unsuitable for resolving proteins or peptides of low molecular weight, which migrate through the

polyacrylamide gel too rapidly. The process also interferes with any subsequent analysis of the protein, such as mass spectrometry (MS) or protein sequencing. In order to reduce these problems there is an ongoing effort to develop methods to supplement or replace it. One such method which show some promise is multidimensional protein identification technology (MudPIT, [Yates 1998; Whitelegge 2002]), which is a gel-free method of protein analysis. The protein mixture of interest is cleaved using proteolytic enzymes and the resulting peptides separated via a series of liquid chromatography separations (multidimensional chromatography). The separated peptides are then fed into a mass spectrometer, and the spectra generated are compared to reference databases to identify the proteins present.

Isozyme staining

Isozymes are enzymes which differ in terms of their amino acid sequences and electrophoretic mobility, and yet catalyse the same reaction. After electrophoretic separation of proteins extracted from a sample, the gel is stained for a particular enzymatic reaction. The staining makes the isozymes catalyse the formation of coloured compounds via a set of coupled reactions. For most meat extracts isozyme staining will lead to several bands on the gel, the relative positions of which are characteristic of the species. Although isozyme patterns are essentially the same for all members within a species, there are distinctive patterns between species (Slattery & Sinclair 1987). Isozyme staining requires that the extracted proteins retain their activity, making it unsuitable for the analysis of processed animal products containing denatured protein (King & Kurth 1982, Slattery & Sinclair 1983). Its application for species identification is therefore limited to raw meat samples (Hsieh 2006).

1.7.1.2 Chromatographic Methods

Chromatography is a sophisticated method for separating mixtures of two or more compounds. Separation is achieved by the distribution of the mixture between two phases: the stationary and mobile phase. Chromatography works on the principle that different compounds will have different solubility and adsorption to the different phases between which they are partitioned. Chromatographic methods such as gas chromatography (GC), liquid chromatography (LC) and high performance liquid chromatography (HPLC) have been used to identify raw and heat-treated meat samples based on an examination of their protein profiles (Ashoor *et al* 1998; Toorop *et al* 1997). Chromatographic methods are not limited to proteins in terms of the compounds that can be analysed. Indeed, chromatographic methods have been used to analyse compounds as diverse as drugs, plastics, flavourings, foods,

pesticides, tissue extracts, fuels, air samples and water samples (Mohrig *et al* 2002) and have been applied to species identification of meat based on fatty acid composition (Carnegie *et al* 1983; Saeed *et al* 1989). They have also been applied for the analysis of bile acids to identify bear bile used in TAM (Espinoza *et al* 1993; Lin *et al* 1997; Lin *et al* 2000).

Despite requiring very small sample sizes, chromatographic analyses can be highly accurate and precise. However, as with electrophoretic methods, chromatographic methods are less effective in detecting adulterated samples (for example, species in meat mixtures) or processed items (such as cooked meats) due to the increased complexity of the chromatographic patterns (Hui 2006). In addition, the requirements of expensive instruments and laborious sample preparation procedures have restricted their widespread use for regulatory purposes.

Capillary electrophoresis (CE) is a sensitive separation technique combining electrophoretic and chromatographic principles. Sodium dodecyl sulphate polymer-filled capillary gel electrophoresis (CE-SDS) has been applied to species identification and quantification of raw meat (Cota-Rivas & Vallejo-Cordoba 1997; Vallejo-Cordoba & Cota-Rivas 1998). Statistical models for pattern recognition utilising linear discrimination analysis have been developed to interpret the resulting protein profiles, and this is sensitive enough to differentiate between mechanically recovered chicken meat and hand-deboned chicken breast based on differences in their haemoglobin content (Day & Brown 2001). In general, however, this level of sensitivity is not desirable for routine species identification as it only serves to complicate results, especially when mixtures of multiple species or other additives are involved. The major advantages of the technique are fast separation, automation and on-line data analysis; however, investment in expensive instrumentation is required.

1.7.1.3 Immunological Methods

The basis of an immunoassay is the natural (immune) response of living organisms to a foreign agent, or antigen. When an antigen enters an organism it triggers the immune system to produce highly specific antibodies that bind to the antigen. Immunoassays are tests which use antibodies as specific binding agents (or which use antigens for the detection or quantification of specific antibodies). The specific nature of the antibodies enables them to distinguish between homologous proteins from different species, making them potentially useful tools for species identification.

Agglutination Test

If an antigen is particulate, the reaction of the antigen with its specific antibody can be detected by agglutination of the antigen. Such tests can be qualitative or quantitative. In qualitative tests the antibody is mixed with the test sample, and the presence of antigen is indicated by agglutination of the particulate antigen. In quantitative tests, serial dilutions of the sample are made and then a fixed amount of antigen added to each dilution. Agglutination techniques, although fairly crude, have been successfully applied to species identification, for example of molluscs and crustaceans (e.g. Suzuki *et al* 1981). A disadvantage of the technique is that when the concentration of antibody is high (i.e. at lower dilutions of antigen) the antibody excess results in very small complexes which do not clump together to form visible agglutination. This is known as the 'prozone' effect, and results in the test giving a false negative.

Precipitation Methods

Another simple immunological method is immunodiffusion, whereby antibodies and test sample diffuse towards each other in a gel. When they meet, if the sample contains the antigen the antibody will bind to it and precipitation will occur, which is easily visualised. Although not very sensitive, this method works well if there is a reasonable amount of antibody or antigen. In radial immunoprecipitation, the antibody is incorporated into an agar gel and different dilutions of the test sample are placed in wells punched into the set gel. Any antigen in the sample diffuses into the gel and reacts with the antibody forming a ring of precipitate around the hole. The diameter of the ring is proportional to the log concentration of the antigen, allowing a quantitative analysis of the amount of antigen in the sample.

A variation on this technique is immunoelectrophoresis, whereby a mixture of antigens is placed in a well on an agar plate and then separated according to charge by electrophoresis. A trough is then cut in the gel and antibodies are added which diffuse into the agar. Precipitation lines form where any antibody/antigen reactions occur. This test is mainly qualitative, although an indication of quantity is given by the thickness of the line. In countercurrent electrophoresis the antibody and test sample are run towards each other. If any antigen is present a precipitation line occurs where they meet. The main advantage of this technique is its speed; however, it only works in conditions where the antigen and antibody have opposite charges. Precipitation methods have been successfully applied to species identification of food products

(e.g. Fugate and Penn 1971; Swart and Wilks 1982) although they have largely been replaced by more sensitive techniques.

Radioimmunoassay and Enzyme Linked Immunosorbent Assay

In these types of assay, the antigen or antibody is labelled and during the test the activity of the label is measured. Radioimmunoassays (RIA) are assays based on the measurement of radioactivity associated with immune complexes, whereas enzyme linked immunosorbent assays (ELISAs) are those that are based on the measurement of an enzymatic reaction associated with immune complexes.

There are three main types of ELISA – competitive, sandwich (indirect) and antibody capture. Antibody capture methods are primarily used to detect antibodies of specific immunoglobulin subclasses. In competitive formats, unlabelled analyte (usually the antigen) in the test sample is measured by its ability to compete with labelled antigen in the immunoassay. The unlabelled antigen blocks the ability of the labelled antigen to bind as it occupies binding sites on the antibodies. Therefore the less label measured in the assay, the more unlabelled antigen (from the test sample) is present. In non-competitive (sandwich) assays the antigen is bound to a solid support (e.g. microparticles, beads, microtitre plates). The test sample is added, and any antigen present binds to the bound antibodies. Enzyme-labelled antibodies are then introduced, which bind to the antigen bound to the immobilised antibodies. After the excess labelled antibody has been washed away, a substrate is added which reacts with the enzyme label to give a colour change. The colour intensity is proportional to the amount of label, which in turn is proportional to the amount of antigen in the sample. Non-competitive assays give the highest level of assay sensitivity and specificity of all formats.

Immunological methods provide rapid, sensitive, specific and cost effective analyses. Furthermore a particular immunoassay format eliminates the need for trained personnel. Lateral flow immunoassays (LFIAs) are rapidly becoming popular diagnostic tools, a familiar example of which is the home pregnancy test. In brief, the LFIA device consists of a strip of porous membrane, with antibodies immobilised at predetermined positions. A sample containing target antigen is mixed with a buffer containing antibodies bound to reporter molecules (such as latex particles, colloidal gold, carbon black or dyed polystyrene). Antigen binds to the antibodies and hence become labelled by the reporter molecule. The sample is then introduced to the membrane at one end and flows along it by capillary action. As the mixture flows over

the capture zones, antigen particles bind to the antibodies bound to the membrane. As the antigen particles are now labelled with a reporter molecule, a positive test is indicated by a visible line at the capture zone.

LFIA devices provide a quick (generally under 10 minutes), simple method of detecting the presence of an antigen in a sample. By targeting a species-specific protein, they could provide a valuable tool for law enforcement officers to use for species identification of wildlife products, provided that the antigen protein has not become denatured during processing of the item. However, lateral flow devices tend to give a higher number of 'false-positives' than the same assays in other formats (Wong & Tse 2008).

Immunological techniques have been applied to the species identification of food stuffs (e.g. Hayden 1981; Hitchcock *et al* 1981; Whittaker *et al* 1983; Asensio *et al* 2003; Kim *et al* 2004). With careful selection of the target antigen, species identification can be successfully carried out even on highly processed samples. For example, targeting antibodies for thermostable muscle proteins can allow the species identification of cooked and even autoclaved meat products (Kang'ethe & Gathuma 1987). Immunoassays do, however, have some disadvantages. Due to their design, sample contaminants can interfere with the antigen-antibody reaction and produce false-positive results. Furthermore if the target protein is highly conserved, it is possible that cross reactivity will occur with the same protein in a different species.

1.7.2 DNA-Based Methods of Species Identification

Certain factors limit the use of proteins for species identification. For example, they degrade rapidly under stress conditions, hence protein-based methods are often unsuitable for the analysis of treated commercial animal products due to manufacturing processes which denature the proteins used for identification (Pereira *et al* 2008). Electrophoretic and immunochemical analyses are often unable to distinguish between closely related species due to issues related to cross-reactivity (Hui 2006) and are often unsuitable for the analysis of processed animal products, as heating and other treatments can denature the target protein and lead to a subsequent loss of analytical specificity.

DNA offers several advantages over protein for species identification. Studies on ancient DNA (fossil bones, ancient organic remains etc.), human forensics (studying DNA from hairs, saliva, blood, semen etc), non-invasive ecological studies (animal

hair, faeces etc) and food authentication (cooked, tinned, processed) repeatedly demonstrate that DNA is generally more resistant and thermostable than proteins. DNA is also present in all tissue types and is more variable than proteins, due to the degenerative nature of the genetic code and the presence of multiple non-coding regions within genomes. Typically high levels of variability allow discrimination at the species, subspecies, population and even individual level. The polymerase chain reaction (PCR) enables *in vitro* amplification of target DNA even from minute and degraded biological traces, enabling analysable DNA to be recovered from manipulated organic products (e.g. rhino horn and ivory ornaments, traditional medicines, cooked, canned or salted food stuffs and so on) and enables minimally-destructive testing of wild life products. The following section will discuss some DNA-based techniques which have been applied to species identification of animal parts and derivatives.

1.7.2.1 Analysis of Mitochondrial DNA

For species identification of wildlife parts and derivatives, the genes usually targeted for sequencing are mitochondrial (mt) in origin. The advantages of using mtDNA in forensic investigations have been demonstrated by several studies (e.g. Allard *et al* 2002; Budowle *et al* 2004; Wilson & Allard 2004) and although these studies focus on the use of mtDNA for the purposes of human identification, the advantages discussed are applicable to wildlife forensics.

The mitochondrial genome in higher vertebrates is typically 15-20 Kb long. With few exceptions, animal mitochondrial genomes contain the same thirty seven genes – including two for rRNAs, thirteen for proteins and twenty two for tRNAs – and in higher vertebrates the order of the genes shows very little variation (Boore 1999). Despite the number and order of the genes being highly conserved across species, mitochondrial genes exhibit extensive interspecific variation, mostly in the form of single nucleotide polymorphisms (SNPs), which can be exploited for species identification. mtDNA is universally present in the mitochondrial organelles of higher organisms, and exists in up to 10,000 copies per cell (Bogenhagen & Clayton 1974) compared to nuclear DNA, which generally has just two copies per cell. mtDNA is therefore more likely to survive in long-dead or highly processed samples. In most species mtDNA is maternally inherited so each individual has only one allele, and the genome doesn't undergo any recombination – features which make mtDNA easier to study and straightforward to work with. A consequence of the maternal transmission is that the effective population size for mtDNA is smaller than that of nuclear DNA.

mtDNA variation is therefore more sensitive to population events such as bottlenecks and hybridisation. Furthermore the mutation rate of mtDNA is higher than that of nuclear DNA. As mitochondria are the sites of cellular respiration, mtDNA is particularly susceptible to damage caused by reactive oxygen species generated by the respiratory chain. This damage, together with the low fidelity of mtDNA polymerase and the inferior DNA repair mechanisms of mtDNA compared to those of nDNA (Kunkel & Loeb 1981; Bogenhagen 1999; Croteau *et al* 1999) mean that the mutation rate of mtDNA can be up to 10 times higher than that of nuclear DNA. Different regions within mtDNA evolve at different rates, with certain regions evolving fast enough to distinguish between closely related species, and even to subspecies level.

Since the introduction of PCR, three methods have come to dominate molecular species identification, and form the basis of over 90% of all studies published on the subject: restriction fragment length polymorphism of PCR products (PCR-RFLP), species-specific PCR and nucleotide sequencing (Teletchea *et al* 2005). PCR-RFLP and species-specific PCR studies are so abundant partly due to the short development times required to design molecular markers but also the low cost and straightforward results obtained from these methods. Nucleotide sequencing studies are popular as this technique provides definitive species identification.

1.7.2.2 DNA nucleotide sequencing

Not long after the development of PCR, methods became available for deducing the nucleotide sequences of the amplified DNA (Gyllensten & Erlich 1988). Studies of sequences recovered from different species revealed highly conserved regions within genomes, enabling the design of 'universal' primers which can be used to amplify homologous regions of the mitochondrial genome from a broad range of taxa (e.g. Kocher *et al* 1989). The combination of PCR/direct sequence analysis allows the identification of the species origin of biological samples (Bartlett & Davidson 1992). DNA is extracted from the sample, and then a specific segment of the isolated DNA is amplified using either universal or species-specific primers (e.g. Kocher *et al* 1989). The nucleotide sequence of the amplified DNA is determined, and then a comparison of the derived sequence is made with reference sequences to obtain a species identification.

For a DNA locus to be of value for species identification, it must show a high degree of interspecific variation, such that closely related species can be distinguished, and

low levels of intraspecific variation to confer confidence in the species designation. The target sequence must be long enough to encompass informative differences between species, but short enough to be recovered from degraded and processed samples. Protein coding regions are preferable as they display less variability than non-coding sequences within a species. Furthermore errors can often be detected by translating the nucleotide sequence and comparing the inferred amino acid sequence with known amino acid sequences of the gene.

The sequences of mitochondrial genes such as cytochrome *b* and 12s rRNA from representatives of many species have been deposited in public databases, such as EMBL (www.ebi.ac.uk) or GenBank® (www.ncbi.nih.gov) (Benson *et al* 2002). Universal primers have been developed for both cyt *b* and 12s (e.g. Kocher *et al* 1989; Irwin *et al* 1991; Verma & Singh 2003) enabling the species identification of unknown samples. A portion of the gene is amplified by PCR using universal primers, the amplified fragment is sequenced and the sequence compared to sequences held in the databases using basic local alignment search tools (BLAST) (e.g. Altschul *et al* 1990; Altschul *et al* 1997). Such comparisons display the closest alignment and also sequences with less homology. An exact or close match in sequence similarity is considered evidence of species identity (e.g. Baker & Palumbi 1994; Baker *et al* 1996), whereby a 'close match' refers to the situation where the difference between the sample and reference sequences is within the expected intraspecific variation of the group under consideration. For example, in a study of the cytochrome *b* gene from rodents and bats, genetic distance values <2% are indicative of intraspecific variation, values between 2 and 11% are indicative of conspecific populations or potential separate species, and values of >11% are considered to represent interspecific variation (Bradley & Baker 2001).

Case reports have been published for the species identification by sequencing of animal parts from a number of protected species, including tiger (Wan & Fang 2003), rhino (Hsieh *et al* 2003) roe deer (An *et al* 2007), snakes (Wong *et al* 2004) game birds (Tandon *et al* 2003) and seahorses (Sanders *et al* 2008). DNA sequencing has also been applied to the identification of cooked and highly processed meat products (e.g. Unseld *et al* 1995; Hsieh *et al* 2005). A disadvantage of using DNA sequencing is that it is only really useful if the species has previously been sequenced for that particular gene, and the sequence deposited in a database. Although vast amounts of sequence data are available for comparison, in the case of bushmeat, it is possible that hunters, who follow loggers deep into undisturbed pristine forests, will encounter

and kill undiscovered species (Peterson 2003). Traditional markets are a rich source of discovery for new species, especially in the biodiversity 'hot spots' of Southeast Asia, an area experiencing explosive growth in unregulated wildlife trade. In the last few years, five new species of ungulates have been discovered or rediscovered through collections of trophies from local hunters or displays in traditional markets of Vietnam or Laos (Groves *et al* 1997; Giao *et al* 1998; MacKinnon 2000; although see Robins *et al* 2006) and Burma/Myanmar (Amato *et al* 1999). A new species of striped rabbit (*Nesolagus timminsi*) was first observed for sale in a market in a rural town in Laos (Surridge *et al* 1999) and a species of rodent, *Laonastes aenigmamus*, the surviving member of a family previously believed to be extinct, was found for sale as wild meat in a traditional market in Laos (Jenkins *et al* 2004; Huchon *et al* 2007). Artisanal and commercial fisheries markets are another likely source of new species, as demonstrated by the discovery of the Indonesian coelacanth *Latimeria* sp. in a Sulawesi fish market (Erdmann *et al* 1998).

Where no sequence match can be made, a phylogenetic approach can be implemented which infers the species identity of a sequence from those of other members of a clade on a phylogenetic tree (Baker & Palumbi 1994, Baker *et al* 1996). The test sequence is grouped, by phylogenetic reconstruction, with the most closely related reference sequences. The resulting reconstruction is usually represented as a tree, with closely related sequences forming neighbouring branches. One or more distantly related species are used as outgroups to guard against misclassification error, and the strength of support for the phylogenetic groupings is evaluated by bootstrap resampling of the sequence data. The relative support for a branch or grouping in the tree is shown as the percentage agreement from a large number of bootstrap simulations. Species identification is considered only if the sequence is nested within the range of reference sequences for a given species (Baker *et al* 1996). In situations where high intraspecific variation or low interspecific variation is present, a test sequence could group with the next most closely related species as a result of sampling error. If a test sequence is intermediate between two groups of reference sequences, it could be a related species or subspecies that is not represented in the reference database.

Although public databases facilitate genetic techniques for identifying vertebrate species, it is assumed that each deposited sequence is error-free and has a correct species label. The public archive of GenBank introduces uncertainty in both sequence quality (Forster 2003) and identity. Phylogenetic analyses make it clear

that error-free publications on mtDNA sequences are extremely rare (Bandelt *et al* 2001; 2002). Indeed, a survey of papers published in a range of journals found erroneous mtDNA sequences in 58.4% of cases (Forster 2003). Sequence-based identification, achieved by similarity-searches using BLAST is essentially a 'closest wins' approach, as there are no clear criteria for dealing with conflicting results. Species representation in such databases is also biased, reflecting research priorities, which introduces further uncertainty into such identifications (Cipriano 2004).

Recently there has been increasing interest in DNA barcoding, which aims to use sequence diversity in short, standardized gene regions for species identification (Ratnasingham & Hebert 2007). The primary barcode sequence for members of the animal kingdom is a 648 bp region of the cytochrome oxidase subunit I (COI) gene (Herbert *et al* 2003ab). The Consortium for the Barcode of Life (CBOL) aims to create a sequence library for all eukaryotic life (Marshall 2005). Submission of a genetic sequence must adhere to formal guidelines, such as the sequence must meet specific quality standards and be derived from a known specimen held in a major collection whose taxonomic assessment can be reviewed if necessary (Hanner 2005). These guidelines allow confidence in the sequences generated as part of DNA barcoding projects, and given the anticipated growth of COI data as a result of such projects, COI promises to be a valuable taxonomic identification tool. With this in mind, this marker has been validated for use in forensic genetic species identification, and was found to consistently identify species where authenticated reference sequence data exists (Dawnay *et al* 2007).

Targeting a single DNA region can be problematic if a polymorphism in a primer binding site causes amplification failure and a null result. In cases where the polymorphisms have been identified, degenerate primers can be designed to circumvent this problem. Single-gene approaches are also vulnerable to the effects of stochastic sequence variation, recombination and horizontal gene transfer. A solution to overcome some limitations of single-gene approaches is the use of multi-locus sequence analysis (MLSA). Here, the sequences of several protein-coding genes are determined and concatenated. The concatenated sequences can then be used to construct a phylogenetic tree, and species identification determined by assessing the clustering pattern of a test sample with related species. MLSA has been successfully used for the identification of closely related prokaryotes (Gevers *et al* 2005), although the main disadvantage of this approach is that it requires the sequencing of several genomic regions, which can be costly and time consuming.

Processed animal parts and derivatives often contain DNA from more than one species, either through contamination (e.g. by human cellular material) or by the presence of more than one species in a manufactured product. Amplifying DNA from such samples using universal primers will result in mixed sequences being amplified. DNA sequencing methods do not allow the discrimination and identification of such mixed sequences, unless fragments are cloned before sequencing to separate each molecule of DNA. An alternative approach is to target individual species within the mixture. To ensure that only the species of interest is amplified by the PCR, the analysis needs to be designed in such a way as to prevent co-amplification of the undesired species. For example, PCR primers can be designed to mis-match the human DNA sequence. This technique has been successfully applied to the identification of highly processed ivory and rhino horn products (Bollongino & Burger 2003).

1.7.2.3 PCR-based Approaches

DNA sequencing is an expensive and time-consuming process. Despite the definitive nature of the results, sequencing is not suitable for high throughput sample processing which is often required for effective CITES enforcement. Such constraints have led to the development of techniques which allow species identification from PCR products without the need for sequencing.

Restriction Fragment Length Polymorphisms (RFLPs)

In RFLP analysis, genomic DNA is digested with one or more restriction enzymes (endonucleases), to generate a set of variable length fragments which can be separated according to their molecular size by gel electrophoresis (Botstein *et al* 1980). As a result of the unique genomic distribution of recognition sites and the distance between them, each species has a distinctive RFLP profile. Early RFLP assays were performed without any amplification step on whole genomic DNA or isolated mitochondrial DNA, and, apart from utilising labelled DNA probes, didn't require any sophisticated equipment or prior knowledge of the sequences of the species being investigated. However they required large amounts of good quality DNA to be effective. The advent of PCR and it's combination with RFLP techniques has enabled the technique to be routinely applied to species identification, and the amplification step means that only minute amounts of starting material is required. Some prior sequence information is required, however, in order to design the PCR primers.

PCR-RFLP has advantages over DNA sequencing such as lower cost, rapid analysis and has been applied to species identification of a variety of animal products (e.g. Wolf *et al* 1999; Cocolin *et al* 2006). Generally a short fragment is amplified – usually from the cytochrome b gene – and subsequently digested. A major disadvantage is the possible existence of intraspecific mutations at restriction sites that can lead to false results due to the gain or loss of restriction fragments. This method relies on just a few informative DNA sequence positions meaning that several restriction enzymes are usually required to achieve a correct identification. In these situations the use of different enzymes generates more highly complex RFLP patterns which may be difficult to interpret accurately, although this problem can be negated by performing a single PCR followed by separate enzyme digests.

An additional problem can occur when analysing genes found in multiple copies within a genome. The intragenomic sequence heterogeneity of certain multigene families (such as rRNA genes) can lead to unexpected RFLP patterns and ambiguous results. However multiple-copy rRNA genes undergo concerted evolution such that sequences of all gene copies are usually very similar within an organism (Liao 2000). The potential presence of two or more different mtDNA sequences in the same cell or individual (heteroplasmy) should also be taken into consideration when analysing RFLP profiles.

A recent improvement to the PCR-RFLP approach is the use of lab-on-a-chip technology, whereby conventional capillary electrophoresis technology is incorporated into an easy-to-use chip-based (LabChip®, Agilent Technologies) format. The chips are small ($3cm^2$), disposable, single-use units containing etched capillaries attached directly to sample loading wells. DNA fragments are separated by capillary electrophoresis and detected using laser-induced fluorescence, allowing fairly accurate sizing and quantification of individual DNA fragments, which gives the system a significant advantage over conventional gel-based approaches in terms of ease of use, speed, safety and reduced costs of consumables. These advantages make this system ideal for further development of species identification based on PCR-RFLP or other DNA profiling techniques (Dooley *et al* 2005).

Species-specific PCR

PCR primers can be designed so that, under stringent PCR conditions, only DNA from the target species will be amplified. By visualising PCR products on an agarose gel, the presence or absence of the target species can be seen without the need for

additional sequencing steps. Although useful for analyses where universal primers are unsuitable, due to the presence of multiple species DNA in a sample, species-specific primer sets are limited in that they target a set number of species at a time due to technical constraints and/or require *a priori* knowledge of the species potentially present in the mixture (e.g. Yan *et al.* 2005; Imaizumi *et al* 2007; Tobe & Linacre 2007; Peppin *et al* 2008). A positive result may give an idea about the presence of a particular species, but a negative result gives no information about the origin of a sample. The specificity and sensitivity of this approach can be enhanced by performing a nested PCR, in which the target region is first amplified with an outer primer pair followed by a second amplification using an internal primer pair.

Multiplex PCR

PCR-RFLP and DNA sequencing are often of limited value when multiple species are present in the sample (Hoelzel 2001). Furthermore they typically require multiple-step, post-PCR processing, and are considered too time consuming and expensive for use in routine monitoring or large-scale market surveys. However such monitoring exercises are often essential elements of enforcement efforts, for example monitoring catches or trade in marine organisms. Multiplex PCR (Chamberlain *et al* 1988) contains multiple primer pairs within a single reaction, allowing the amplification of multiple sequences within that reaction.

Multiplex PCR has been applied to the identification of shark parts. The status of the world's commercially fished sharks is of international concern. As different shark species respond differently to exploitation pressure, management and conservation efforts on a species specific basis are imperative. However the morphological similarities of many commercially harvested species, coupled with onboard processing practices, mean that it is difficult to identify sharks and monitor catch on a species-specific basis. The problem is exacerbated by the practise of shark 'finning' whereby only the detached fins are kept and subsequently traded, typically in dried form. Accurate species identification of shark parts is essential if fisheries regulations are to be enforced. To facilitate efficient species identification of shark body parts a technique has been developed utilising shark "universal" and species-specific PCR primers in a high-density multiplex format to simultaneously discriminate between multiple shark species with a single-tube PCR (Shivji *et al* 2003). The species-specific primers are based on DNA differences between species in the nuclear ribosomal internal transcribed spacer 2 and mitochondrial cyt *b* loci, which are highly conserved within shark species. Each species-specific primer is designed to anneal

to these loci in regions producing a diagnostic size amplicon, which can be identified by electrophoresis.

Random amplified Polymorphic DNA (RAPD)

RAPD profiles are generated by the random PCR amplification of DNA segments using short primers of arbitrary nucleotide sequence (Williams *et al* 1990). Amplification products are generated when two RAPD primers anneal within a few thousands bases of each other in the correct orientation, and species can be characterised by specific banding pattern in an electrophoretic gel resulting from the different genomic location of primer-binding sites. The banding pattern generated is consistent for the same primer, DNA and conditions used. The method is simple to set up as no prior knowledge of the genetic make-up of the organism is required. The primer is chosen at random and the information generated can discriminate the species and even at the individual level. Furthermore, as the approach is PCR-based only a very small amount of starting material is required. RAPDs have been used for species identification in a range of taxa, including fish (Partis & Wells 1996), birds (Haig *et al* 1997), mammals (Lee & Chang 1994) and gastropods (Klinbunga *et al* 2004). However, the reproducibility of the technique is questionable (Jonas *et al* 2000) and is affected by template concentration, PCR and electrophoretic settings etc. Carefully developed laboratory protocols must be followed to ensure reproducibility, as an imperfect hybridisation between the primer and the target site could result in a completely different banding profile. The RAPD method, as well as other fingerprinting techniques, generates results from mixed samples which can be difficult to interpret. Reproducibility can be improved by using good quality DNA and ensuring the sample is not of mixed origin, however this is rarely possible given the processed nature of most wildlife products.

Amplified Fragment Length Polymorphisms (AFLPs)

The AFLP method (Vos *et al* 1995) combines the reproducibility of restriction fragment analysis with the power of PCR. It is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. This method usually works by digesting a small amount of purified genomic DNA with two or more restriction enzymes. Double stranded oligonucleotide adapters (10-30 bp long) are ligated to the sticky ends of the DNA fragments generated during the restriction digestion. The ligated DNA fragments are then amplified twice under highly stringent conditions by PCR using primers complementary to the adapter and restriction site sequence. These selective primers include additional nucleotides at

their 3' end to reduce the complexity of the mixture of fragments generated. The AFLP technique permits the simultaneous screening of different loci randomly distributed throughout the genome, however it is technically demanding, labour consuming and the interpretation of results may require automated computer analysis. Additionally, AFLP is quite a costly technique since it requires an expensive software package to analyse a large number of AFLP patterns. The information is similar to that obtained by RAPD, however AFLP markers exhibit much higher reproducibility (98-99%, Questiau *et al* 1999) and more clearly evidence Mendelian segregation. This disadvantage of dominance is in part overcome by the fact that hundreds of AFLP bands can be obtained, allowing for true fingerprinting of individuals. AFLP has been successfully applied to the species identification of processed products, for example smoked sturgeon and caviar (Congiu *et al* 2001).

Real-time PCR

Real-time PCR enables the detection of a specific DNA sequence in a sample by measuring the accumulation of amplified products during the PCR using fluorescent technologies (reviewed in Lockley & Bardsley 2000; Mackay 2004; Monis *et al* 2005). There are two common methods of DNA quantification. In the first method, a fluorescent dye (e.g. SYBR Green I [Wittwer *et al* 1997]) which binds to double-stranded DNA (dsDNA) in a sequence-independent manner, is added to the reaction mixture. When the dye binds to dsDNA, its fluorescence increases. During PCR, an increase in dsDNA product leads to an increase in fluorescence intensity which is measured after each cycle. The second method uses sequence-specific probes (e.g. TaqMan probes [Holland *et al* 1991]) and fluorescence resonance technology (FRET). The probes have a fluorescent reporter at one end and a fluorescence-quencher at the opposite end. During PCR, the probes anneal to a target DNA sequence between the forward and reverse PCR primers. As polymerisation of the new DNA strand progresses, the probe is denatured by the DNA polymerase. This causes the fluorescent reporter to be separated from the quencher, resulting in an increase in fluorescence which is then detected and measured. For both methods, if species-specific PCR primers are utilised, the positive signal (increase in fluorescence) indicates the presence of the target species without the need for any additional analysis.

Alternatively, species-specific or universal primers can be used followed by melt-curve analysis (MCA). In MCA, the melting temperature of the amplified fragment is used to determine species identification. The melting temperature (T_m) of a fragment

is determined by the nucleotide content and length of the fragment. It is therefore possible to design primers to generate fragments with species-diagnostic melting temperatures (e.g. Berry & Sarre 2007). Alternatively, universal or group-specific primers can be used (e.g. Harasawa *et al* 2005). Although generally the fragments amplified from different species will be the same size, they may still have different T_m due to interspecific sequence variation.

The majority of DNA-based methods of species identification involve multiple post-PCR manipulations of samples (e.g. gel electrophoresis, sequencing reactions) which not only add time and cost to the processing of samples, but also increase the chances of human error and contamination. Real-time PCR offers advantages over this in that it does not require any post-PCR manipulation of samples. It also offers increased sensitivity by permitting the discrimination of spurious PCR amplifications from non-target DNAs (using TaqMan probes), and is a relatively fast SNP genotyping method, with some platforms affording high-throughput automation. Examples of species identification or animal products by real-time PCR include processed food stuffs (Brodmann & Moor 2003) and canned fish (Lopez and Pardo 2005). The most significant disadvantages of real-time PCR methods are the incompatibility of certain platforms with some fluorescent dyes, the restricted multiplex capability and the high cost of most reagents and instrumentation.

DNA Microarrays or DNA Chips

Wildlife products often contain several different species, or several individuals from the same species. Furthermore, they may contain complex mixes of bacteria and fungi, or be contaminated with DNA from other species (e.g. human). Few methods allow the simultaneous identification of multiple species or individuals within a sample. The two methods with the most potential to provide a solution to this problem are (i) cloning and sequencing and (ii) DNA chips (DNA microarray). The cloning approach requires the amplification of DNA extracted from a mixed sample using universal primers followed by cloning to separate each molecule of DNA. The separated molecules are then sequenced using a direct sequencing method, and the generated sequences compared to those in genetic databases. This approach was successfully used to identify five species in a can of pet food labelled 'meat flavour' (Donne-Goussé *et al* 2002), yet despite this ability to identify a wide range of food stuffs the method has rarely been applied. An alternative method, using universal primers and interspecific length polymorphism in the mitochondrial control region, has been applied to the identification of mammals in mixed samples (Pun *et al* 2009), however

many of the diagnostic size fragments were over 500 bp and some over 800 bp in length making it unsuitable for the analysis of highly degraded DNA, as is often found in processed animal products.

A DNA microarray (also known as gene chip, DNA chip or biochip) is a collection of DNA probes attached to a solid surface, usually glass or silicon. Probes can include synthetic oligonucleotides, amplicons or larger DNA/RNA fragments selectively spotted or addressed to individual test sites in the microarray. The microarray is scanned or imaged to obtain a complete hybridisation pattern generated by the release of a fluorescent, chemiluminescent, colorimetric or radioactive signal associated with the binding of the probe to the target DNA sequence (Heller 2002). This technology has huge potential in the field of genomics (e.g. gene expression profiling, mutation detection and analysis) and proteomics (e.g. analysis of protein activities, allergy screening) and, although not much work has been done in this area as yet, the technology also has potential in the field of forensic species identification.

Advances in printing technology have enabled the production of microarrays containing hundreds of thousands of probes (high density micro-arrays may have up to 10^6 test sites on the 1 – 2cm^2 area) revealing the potential to achieve sensitive and high throughput species identifications. It would be possible to create, say, a 'bushmeat chip', incorporating diagnostic markers to unambiguously identify all protected species known to be traded illegally as bushmeat. Development of such mixed-phyla chips has already begun, with promising results (Pfunder *et al* 2004) and this technology promises to be an invaluable tool in the enforcement of CITES regulations. However such chips have long development times, high associated costs, require specialised robotics and imaging equipment that are not generally available in most laboratories, and require advanced bioinformatic tools to reduce the complex data into useful information. Furthermore, such chips only detect a predefined set of taxa, and are therefore uninformative if unexpected species occur in a sample. These factors have limited their application to forensic samples in the past (Teletchea *et al* 2005).

1.7.2.4 Limitations of mtDNA for Species Identification

Given the maternal inheritance of mtDNA, it is expected that only one version of the DNA molecule should be present in each individual. However heteroplasmy – the presence of more than one mtDNA type within a cell – is common throughout the animal kingdom (e.g. Hauswirth & Laipis 1985; Rand 1993). Heteroplasmy arises

either as the result of the male mitochondrion entering the ovum during fertilisation, which occurs in approximately 1 in 10,000 fertilisations (Kobilinsky *et al* 2004), or due to mutations occurring in somatic or oocyte cells (Petri *et al* 1996). In humans, heteroplasmies accumulate in different tissues with increasing age (Calloway *et al* 2000), and specific mtDNA mutations are linked to certain diseases (Wallace 1992; Ozawa 1995). The occurrence of heteroplasmy does not invalidate the use of mitochondrial DNA for forensic casework, and guidelines have been developed to assist accurate analysis (Carracedo *et al* 2000).

Translocated pieces of mitochondrial DNA (known as numts or pseudogenes) are sometimes integrated into the nuclear genome (Lopez *et al* 1994). As whole genome sequencing projects progress, increasingly more numts are being detected in diverse eukaryotic organisms (Bensasson *et al* 2001). In metazoans, numts effectively lose their function upon transfer to the nucleus (Gellissen & Michaelis 1987) and evolve inoperably in a nuclear-specific fashion, in the absence of the strong purifying selection acting upon mitochondrial genes (Schmitz *et al* 2005). The pattern of nucleotide substitution of numts is quite different to that of mitochondrial DNA, due to higher overall rates of molecular evolution and a strong bias toward third codon position in mtDNA (Mundy *et al* 2000). They often contain insertions or deletions (indels) that would cause frameshifts, nonsense mutations and nucleotide substitutions which would result in unique amino acid replacements at sites that are highly conserved in the mtDNA-encoded proteins (Zhang & Hewitt 1996).

Differences between the real mtDNA and pseudogene sequences of up to 18.8% have been reported (Williams & Knowlton 2001). During PCR, numts may be inadvertently amplified in addition to or instead of the authentic target mtDNA, which is a major problem in phylogenetic and population genetic studies employing mitochondrial genes (Bensasson *et al* 2001; Thalmann *et al* 2004). A number of studies have identified methods which reduces the possibility of numt amplification during PCR, and made suggestions of how to check for numts in the amplified product (Bensasson *et al* 2001 and refs therein), however no method can guarantee that numts will not inadvertently be amplified.

Another limitation of mtDNA is that it is not appropriate for identification of species which are able to hybridise. Hybridisation between closely related species (introgression) can occur when species share overlapping habitats, or through human intervention (e.g. Ludwig *et al.* 2003; Redenbach & Taylor 2003). Such

introgressions can cause a mismatch between mtDNA haplotype and species identity (Moritz & Cicero 2004). These mismatches cannot be identified by analysing multiple mitochondrial sequences as mitochondrial genes are all part of single linked locus, and in such situations a combination of nuclear and mitochondrial sequences is recommended (e.g. Bacon *et al* 1999). Although introgressions of nDNA can also occur, this is subject to far greater constraints and occurs differentially across the genome (Avise *et al* 1994).

The issue of hybridisation is particularly relevant to the wildlife trade, as the molecular taxonomy of species can be complicated by market as well as evolutionary forces. Trade in exotic species often places a priority on novelty, providing incentives to breed unusual specimens in captivity and fabricate collection data (Dalton 2003). Over the last two decades, fourteen new species of freshwater turtles have been described from China, ten of which were described from the morphology of specimens purchased through the Hong Kong wildlife trade (Stuart & Parham 2007). Genetic analyses indicate that these 'species' are in fact intergeneric hybrids bred in captivity (Parham *et al* 2001; Stuart & Parham 2007). An improved molecular taxonomy, including a comprehensive reference database, is urgently needed to control trade and direct conservation efforts for 'true' species under threat (Shiping *et al* 2006; Fong *et al* 2007).

1.8 Population Assignment

As wildlife is often managed according to stocks, geographical populations (Dizon *et al* 1992) or on the basis of whether specimens are captive-bred or wild-caught (see section 1.4) it may be necessary to determine the population origin of a specimen in order to secure a prosecution. The ability to determine the origin of specimens will also help the identification of trade routes, and help direct conservation efforts and distribute law-enforcement resources optimally (Goldberg 1997; Primmer *et al* 2000; Baker 2008).

If populations of a species are isolated from one another to the extent that there is effectively no gene flow between them, genetic differences will accumulate over time until the populations are genetically distinct from one another. Various methods of population assignment using genetic data exist, with the most applicable method depending on the level of genetic differentiation between populations, but all require the existence of population data from multiple populations.

1.8.1 Assignment Using mtDNA Phylogenies

The hypervariable mtDNA control region or 'D-loop' is often used as a marker in geographic origin identification, with individual control region sequences ('haplotypes') corresponding to specific populations (e.g. Goldberg 1997; Wu *et al* 2005). Assignment using gene sequences employs the same approach as species identification using sequence data to reconstruct phylogenetic trees. By comparing a sample sequence to population reference sequences, it is possible to assign the unknown sample to a population or geographical area (e.g. Baker & Palumbi 1994; Goldberg 1997; Dizon *et al* 2000), assuming that all potential source populations have been sampled.

mtDNA phylogenies are only suitable for assignment when populations are highly divergent; the application of this method is limited in species with low levels of genetic differentiation (e.g. Baker *et al* 1996, Dizon *et al* 2000). Furthermore most assignment tests crudely assign a specimen to the stock in which the individual's haplotype is most common. Thus, incorrect assignment may occur when a haplotype is common in a rare population but less common in an abundant population, as the unknown sample will be assigned to the rare population even if there are more individuals with that haplotype in the abundant population (Taylor 1997; Dizon *et al* 2000).

1.8.2 Assignment Using Nuclear Loci

In many cases geographical populations or stocks are not phylogenetically distinct or delimited by diagnostic molecular characters. In the absence of sufficient mitochondrial DNA variation, variable markers from the nuclear genome may be utilised, such as SNPs or microsatellites. Although these markers show some discrete differences, individual alleles are often distributed across populations, and differentiation can only be achieved on the basis of differing allele frequencies among potential source populations (Manel *et al* 2005). With extensive sampling of a species throughout its range, the frequency of the observed alleles can be used to characterise the genetic structure of the various populations, and multilocus assignment tests then used to estimate the origin of an individual specimen.

A wide range of analytical methods are available for assignment, which vary in terms of their statistical basis and underlying assumptions (Manel *et al* 2005). Assignment methods can address two basic types of problem: classification and clustering, with classification being the most relevant to forensic investigation. For forensic analyses,

a method that enables both the prosecution and defence hypotheses to be evaluated must be applied (Ogden *et al* 2009). Although identifying the most likely source population is desirable, the potential to exclude a specific population of origin can also be very informative (e.g. Primmer *et al* 2000). This is particularly true for endangered species as sampling opportunities are limited, making it difficult to establish comprehensive reference databases (Ogden *et al* 2009), which in turn means that specimens cannot be assigned confidently to the source population (Schwarz & Karl 2008).

'Inclusion' or 'exclusion' approaches may be used for population assignment. Inclusion approaches try to determine which population an unknown sample derives from, and assume that the source population is represented in the reference database. Exclusion approaches assess which population the sample did *not* come from, and assume that the source population has not been sampled. Population assignment using exclusion has been reported (e.g. Primmer *et al* 2000; Schwenke *et al* 2006). Exclusion approaches are generally more appropriate, as it is rare that a researcher can be confident that all populations of a species have been adequately sampled (Cornuet *et al* 1999).

Assignment methods are implemented using distance, frequentist and/or likelihood (maximum likelihood [ML] or Bayesian analysis) statistical approaches. Distance approaches use a measure of genetic distance to the 'closest population', and are considered to be more suited to exploratory data analysis than to fine statistical inference (Pritchard *et al* 2000). The frequency approach assigns an individual to the population in which the individuals' genotype is most likely to occur (Paetkau *et al* 1995), and assumes that all loci are independent, are in Hardy-Weinberg equilibrium, and that the allele frequencies deduced from the population samples are close to their exact values (Cournuet *et al* 1999). This last assumption prevents population assignment when the query specimen carries a rare allele that was not detected during the sampling of the source population. In this case a likelihood of zero would be calculated, eliminating the true population from the analysis. However adjustments to the technique can be made to avoid this problem (Paetkau *et al* 1995, Cornuet *et al* 1999).

Likelihood methods assume that the observed data arose from a probabilistic model with unknown parameters, and aim to use the data to estimate these parameters, and assess the degree of uncertainty associated with these estimates (Manel *et al*

2005). ML methods produce point estimates of model parameters that maximise the likelihood function, whereas Bayesian methods produce posterior distributions for model parameters (Manel *et al* 2005). A number of studies demonstrate that the Bayesian approach is the most effective for population assignment and phylogeny interpretation (e.g. Cornuet *et al* 1999; Huelsenbeck *et al* 2001; Corander *et al* 2003; Wasser *et al* 2004; Hauser *et al* 2006). The main problem with population assignment in a forensic context is that there has been little or no forensic validation of the various algorithms used, and a general lack of understanding exists regarding how the methods perform when the assumptions of the models are violated, as often happens with wildlife species. As a result, there is no formal consensus over which approach or model to use, and there is no precedent from human applications to aid model selection (Ogden *et al* 2009).

In species where gene exchange between populations is high, there may be no discernable differences in allele frequencies between populations, making assignment tests redundant. This is common where populations are defined by non-biological criteria, such as country borders. In these situations non-genetic approaches such as stable isotope analysis offer an appropriate alternative (e.g. Pain *et al* 2004). The use of this technique to trace the origin of wildlife is based on the fact that stable isotope signatures in animal tissues reflect those of local food webs (e.g. Hobson 1999). Isotopic signatures of food webs can vary spatially due to biogeochemical processes and these 'signatures' are passed on to consumers feeding in those food webs (DeNiro & Epstein 1978, 1981). Animals that move between isotopically distinct food webs can retain information on previous feeding location, and variation in elemental turnover rates between tissues allows analysis to be performed at different temporal scales (Hobson 1999). Applications of the technique to population assignment include identification of the wintering sites of migratory birds (e.g. collared flycatcher [Hjernquist *et al* 2009]), identification of the geographic origin of elephant ivory (Van der Merwe *et al* 1990) and rhinoceros horn (Hall-Martin *et al* 1993).

1.9 Individual Identification

In some instances, it may be necessary to demonstrate that an animal part or derivative originates from a particular individual. For example, it may be necessary to link a confiscated horn, tusk or skin to a particular carcass, especially if a park ranger has been killed or injured during the incident. The use of DNA profiling for the identification of individuals has revolutionised human forensic analysis over the last

20 years, and more recently has been applied to non-human forensics. DNA profiling works by targeting markers which exhibit high levels of intraspecific variability. Several markers are analysed to generate a 'profile' of alleles for each individual. If two samples have different profiles, the hypothesis that they originated from the same individual can be excluded. If the two samples share the same profile, it is possible that they originated from the same individual, and statistical techniques can be used to assess the probability that the samples have the same profile by chance or because they did indeed originate from the same individual.

Various genetic markers have been used for DNA profiling, with short tandem repeat (STR) profiling currently being the most powerful. STR profiling for humans is well established, however the development of individual profiling techniques for wildlife has been limited by the need to identify and validate new markers for virtually every individual species, as STR loci must fulfil certain criteria prior to their application in forensic casework. Loci used in forensic human identification have undergone extensive validation and characterisation which has led to the production of guidelines and standard operating procedures to aid the interpretation of STR profile data (Gill *et al* 1997; Bär *et al* 1997; Gill *et al* 1998; Gill *et al* 2006). As yet, there are no specific guidelines for the development of forensic STR profiling systems in wildlife species (but see Dawnay *et al* 2008). Many primer notes detailing the development of STR markers state forensic investigation as a potential application (e.g. Jones *et al* 2002; Kurushima *et al* 2006), yet without thorough validation, these markers cannot be used to generate useful evidence. Only a handful of studies have validated non-human STR profiling systems to enable their application to forensic casework (e.g. Butler *et al* 2002; Halverson & Basten 2005; Dawnay *et al* 2008; Ogden *et al* 2008; Dawnay *et al* 2009) following the guidelines established for human profiling systems. However it has emerged from these studies that certain forensic recommendations cannot easily be followed when working with certain species (Dawnay *et al* 2008), highlighting the need for greater attention to be paid to the issues concerning validation of such systems (Ogden *et al* 2008).

When matching STR profiles are found, the probability of observing the match by chance is calculated to assess the strength of the evidence. Forensic calculations are conventionally presented as either the probability of a random match (called the match probability, calculated from the frequencies of DNA markers in the database) or the likelihood ratio (LR). The LR is the ratio of the probability of a match if the two STR profiles originate from the same individual, to the probability of a match if they

come from different individuals. Since the probability of a match if the profiles come from the same individual is 1 (assuming the analysis was error-free), the likelihood ratio is the reciprocal of the match probability. Such probabilities were previously calculated under the assumption of independence of profiles (Ayres & Overall 1999), however due to at least some level of shared ancestry in populations, STR profiles are not independent (Donnelly 1995). The assumption of independence can severely affect the calculated match probability, causing an overestimated of the strength of DNA evidence (Balding & Nichols 1994). This calculation is therefore amended to account for population stratification by including the population genetic coefficient θ (also known as F_{ST}) (Evett & Weir 1998) into the match probability.

Early models also assumed random mating within the population (Balding & Nichols 1994), which may be violated in some species or populations. Inbred populations generally show a reduction in the number of heterozygotes when compared to Hardy-Weinberg expectations which subsequently decreases the match probability when a heterozygote DNA profile is observed and increases the match probability when a homozygote DNA profile is observed. To account for the occurrence of non-random mating the value of f (also known as F_{IS}) may be included in the match probability (Ayres & Overall 1999; but see also Dawnay 2008).

Wildlife profiling systems are also often limited by difficulties associated with obtaining sufficient reference data. The probability that two matching profiles originate from the same individual is affected by the number and variability of markers in the profile, allele frequencies and how closely related individuals are in the source population (Evett & Weir 1998). Considerable reference population data is needed to evaluate these factors, which can be difficult to acquire in rare or endangered species.

Despite these challenges, systems for individual identification can provide useful tools for monitoring wildlife trade. If genetic samples are collected systematically as part of a regulated hunt or catch, individual identification can be used to track the origins of a product in trade and verify its legitimacy (e.g. Cipriano & Palumbi 1999). Genetic tracking can also be used to describe market dynamics, especially for large species that are butchered before transport (e.g. whale meat, Dalebout *et al* 2002). A formal extension of genetic tracking involves establishing a 'DNA register' of all individuals destined for trade (Dizon *et al* 2000; DeSalle & Amato 2004). The DNA profile of an item can then be compared to the database. The absence of matching

profile in the database would suggest that the item was illegitimate (Dizon *et al* 2000). The ability of DNA registers to identify illegitimate samples has been demonstrated (Palsbøll *et al* 2006). However a number of technical problems have also been highlighted, such as inter-laboratory variation in allele binning, estimates of genotyping errors etc., which would need to be addressed before DNA registers could be implemented for trade regulation. Furthermore, legitimate sale of non-registered specimens could also hamper the effectiveness of DNA registers. For example, the Japanese government has committed to maintaining a genetic register for species taken in it's scientific whaling programme, but the situation is complicated by the sale of products from whales caught as fisheries by-catch and by the long-term storage of products predating the register (Baker *et al* 2007).

1.10 Importance of Validation of Forensic Techniques

Validation is the process by which the ability of a procedure to reliably obtain the desired result is assessed, the conditions under which such results can be obtained are defined and the limitations of the procedure are determined. In addition, the validation process identifies the critical aspects of a procedure which must be carefully controlled and monitored in order to produce robust, reliable and reproducible results (SWGDAM 2003). Since the data generated during validation studies is necessary to assess a method's quality, the importance of validation for forensic techniques cannot be over-emphasised.

There are two main types of validation study – those that establish the validity of the procedure in general (developmental validation) and those that establish that the procedure developed will work effectively when performed in a different laboratory, using different equipment operated by different staff members (internal validation) (Butler 2001). Ideally, validation studies should demonstrate a protocol's range of sensitivities, and include conditions which are too extreme for the protocol to produce an interpretable result. These studies should also illustrate how the method performs on degraded samples. Validation studies are often viewed as tedious and costly in terms of the time and resources required (Butler 2006), but in situations where evidence is challenged, data from validation studies provides the most effective defence of the data. It is also important to ensure that the data produced is robust, reproducible and reliable to prevent unjustified legal consequences for the defendant. Furthermore, unvalidated techniques can waste time, resources and money as they are implemented, utilised and subsequently found to be unfit for purpose. Conversely, laboratories can sometimes spend excessive time and resources

validating techniques; a balance exists between thorough examination of a technique and rapid implementation.

To assist laboratories in achieving an appropriate balance, a number of organisations have produced guidelines to guide validation studies (e.g. DNA Advisory Board [DAB], Scientific Working Group on DNA Analysis Methods [SWGDAM]). These guidelines emphasise that at least 50 samples should be used for internal validation, and these samples can come as part of studies examining some or all of the following: known and non-probative evidence samples, reproducibility and precision, match criteria, sensitivity and stochastic studies, mixture studies, contamination and a qualifying test. Furthermore these guidelines state that the assessment of the limits of new assays or typing technologies should be examined with authentic case samples where possible (SWGDAM 2003).

Each laboratory using a particular procedure follows standard operating procedure (SOPs) that detail the materials and the steps required to successfully complete the experiment. SOPs also list the critical aspects of the assay which must be carefully monitored to ensure the desired result is obtained. SOPs are strictly adhered to when forensic casework is performed to ensure that the integrity of the evidence generated is not compromised.

In addition to validating methods, it is important to validate data contained within the reference databases used in genetic analyses, whether for species identification, geographical designation or population assignment (Parson *et al* 2004). For species identification this is being achieved through initiatives such as CBOL, whereas validation of reference data for population and individual identification relies on accurate inter-laboratory allelic identification. This has somewhat limited the exchange and development of these techniques in wildlife forensics, although reference databases have been successfully created for some species (e.g. Seeb *et al* 2007; Dawnay *et al* 2008), and clear guidelines established for allele nomenclature systems and population data production (Bär *et al* 1997; Lincoln & Carracedo 2000).

1.11 Summary & Aims

Illegal wildlife trade presents a serious threat of zoonotic disease, species extinction and social and economic problems associated with organised criminal elements attracted to this illegal activity. When conducted at unsustainable levels, wildlife trade may undermine efforts to achieve sustainable development and poverty alleviation.

The development of techniques for the analysis of wildlife products (in terms of species identification, geographic origin and individual identification) greatly assist the implementation of CITES and other relevant legislation. Furthermore, such techniques act as powerful deterrents to potential wildlife traffickers, as studies indicate that increased threat of detection is more of a deterrent to criminals than higher penalties (Ehrlich 1973; Avio & Clark 1978). A wide range of techniques have already been applied to the analysis of wildlife products, however these techniques are primarily laboratory-based and costly in terms of time and resources. Furthermore, certain wildlife products prove to be particularly challenging, and the development of new approaches would be very useful.

The aims of this PhD are as follows:

1. To assess the feasibility of using immunoassay technology to produce a field-based kit which could be used by people with little or no scientific training to screen suspected illegal wildlife products and identify those items which warrant closer scrutiny through laboratory-based techniques. The current project will use the illegal trade in bear (Ursidae) parts and derivatives, particularly bear bile used in TAM, as a case study, but the findings will have a much broader application. To complement the field test, genetic methods of analysis will also be developed to enable the results of the field test to be verified.
2. To assess the feasibility of using STR profiling and mtDNA typing for matching evidence samples to the individual of origin, in a species which has recently undergone a severe population bottleneck. Here, we use the illegal trade in horn from white rhinoceros *(Ceratotherium simum*) as a case study.
3. To survey and assess the authenticity of 'exotic' meats sold through UK-based mail order companies. Mislabelling of products has implications for fair trade and consumer choice, as well as more serious implications regarding food allergies and religious preferences.
4. In addressing 1-3 above, a synthesis and critique of approaches to the detection and monitoring of wildlife trade will be considered, as well as the challenges and future opportunities of developing an effective forensic framework for legal enforcement in relation to conservation and management of wildlife trade.

Chapter 2

Development of Methods for the Analysis of Suspected Bear Parts & Derivatives

Publications

Publications resulting from this work (Appendix 4):

Peppin L, McEwing R, Carvalho GR and Ogden R (2008) A DNA-Based approach for the forensic identification of Asiatic black bear (*Ursus thibetanus*) in a traditional Asian medicine. *Journal of Forensic Sciences,* 53(6):1358-1362

Peppin L, McEwing R, Webster S, Rogers A, Nicholls D and Ogden R (2008) Development of a field test for the detection of illegal bear products. *Endangered Species Research,* Special Edition: Forensic Methods in Conservation Research

Chapter 3

Development of DNA-Based Methods for the Analysis of Parts and Derivatives of the White Rhinoceros

Publications

Publications resulting from this work (Appendix 4):

Peppin L, McEwing R, Ogden R, Hermes R, Harper C, Guthrie A and Carvalho GR (2009) Molecular sexing of African rhinoceros. *Conservation Genetics*, Available online April 2009

Chapter 4

Authenticity of Exotic Meats Sold in the UK

Chapter 4:
Authenticity of Exotic Meat Sold in the UK

4.1 Introduction

The UK market for exotic and speciality meat comprises a range of imported products from species such as crocodile, bison, kangaroo and African game species including springbok, kudu and eland (Bicknell 2001; Hoffman & Wiklund 2006). Although relatively small, the market saw significant growth in the post-BSE 1990's as consumers sought safe alternatives to beef, and continues to grow (Bicknell 2001) due to a factors such as:

1) Red meat (especially beef) is increasingly associated with obesity due to its total dietary and saturated fat content (Hoffman & Wiklund 2006). The lower fat, cholesterol and improved fatty acid profiles of exotic and speciality meats compared to domestic meat appeal to customers wishing to adopt healthier lifestyles.
2) Exotic and alternative meats are often produced using organic and natural production (low input) methods (Dransfield 2003; Steenkamp 1997), appealing to consumers concerned about the environment and animal welfare.
3) Increases in tourism and immigration expose individuals to a wide range of food stuffs, and consumers are increasingly willing to experiment with exotic and ethnic foods (e.g. Hoffman *et al* 2003).

As a result of these driving factors, the market for game and exotic meat in the UK was £61 million in 2006, roughly 50% higher than in 2004 (Mintel 2007).

Food labelling regulations require that the species of meat in meat products is accurately declared. Falsification, adulteration and mislabelling of meat products is prohibited for reasons concerning fair trading, consumer protection, religious reasons and public health (Hsieh 2006). Fraudulent or unintentional mislabelling of meat products still, however, exists. Indeed along with alcohol, meat is the prime target of food fraud (Which? 2009).

Under EU law, 'traceability' refers to the ability to track any food, feed, food-producing animal or substance destined for consumption, through all stages of production, processing and distribution. When a risk (such as disease or contamination) is identified, it is vital that products can be traced back to source quickly to isolate the problem and prevent contaminated products from reaching

consumers. Past food crises relating to food safety, such as bovine spongiform encephalopathy (BSE) and dioxin contamination, have illustrated the importance of swift identification and isolation of unsafe foodstuffs, thereby driving the development of food safety legislation (Hobbs *et al* 2002; Roosen 2003). In the event of such crises, traceability helps minimise trade disruption by allowing targeted product withdrawals, and assists the provision of accurate information to the public.

Traceability systems for exotic and alternative meats have been established for products imported into the EU (e.g. Hoffman & Wiklund 2006). However, accurate species labelling of products is essential for traceability systems to function effectively. Furthermore, mislabelling can result in 'risky' items not receiving the necessary checks and therefore posing a risk to public health. For example, all products of animal origin imported into the UK from countries outside the EU must enter at an approved border inspection post (BIP), whereby the accompanying documentation is checked. An 'identity check' is performed to ensure the product matches the details in the documentation, and a physical check on the product itself is performed, which may include laboratory analyses (Defra 2009). Only a percentage of products is subject to the physical check; between 1% and 50% depending on the animal and public health risks associated with the product. If the product is mislabelled, the level of risk associated with the product may be underestimated, which could in turn lead to inadequate testing of the product and, ultimately, a risk to public health.

The need for accurate labelling of meat products is perhaps even more pertinent for exotic meats as customers are likely to be unfamiliar with the products, and less able to identify mislabelled products themselves. For example, most consumers in the UK could easily distinguish between lamb and beef, but may not be able to distinguish between kudu and springbok.

The aim here was to assess the authenticity of exotic meat products sold within the UK, by sequencing portions of the cytochrome *b* gene (cyt *b*) to obtain a species identification, and comparing this to the designated identity.

4.1.2 Method

Samples

Meat products were purchased from two UK-based suppliers, who will be referred to here as Company X and Company Y (Fig 4.1 and 4.2). The products purchased from

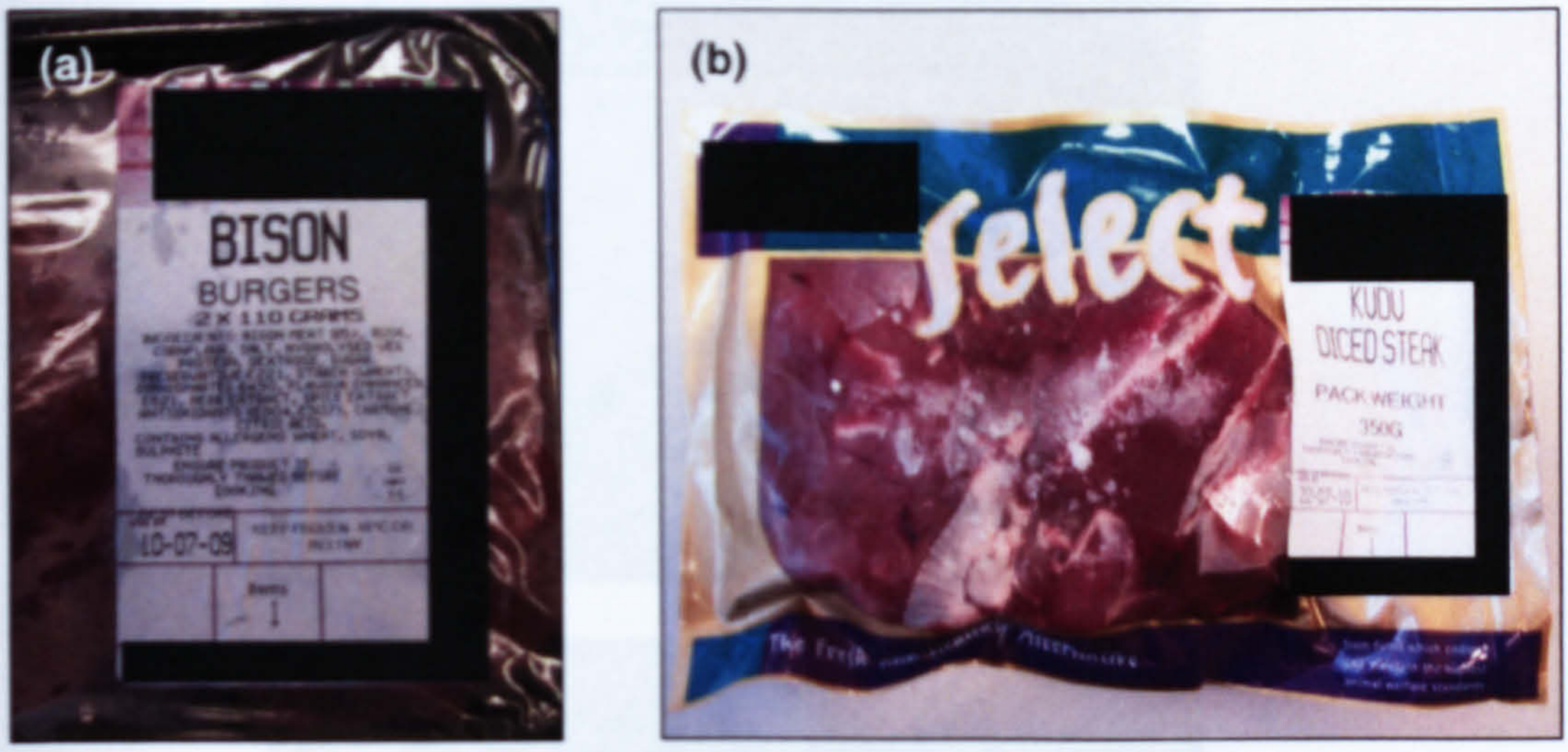

Fig 4.1 Exotic meat products purchased from Company X (a) bison burgers (ingredients state 95% bison meat; no other animal meat listed) (b) Diced kudu steak (c) diced springbuck steak (d) impala burgers (ingredients state 97% impala steak; no other animal meat listed)

Fig 4.2 Exotic meat products purchased from Company Y (a) kudu steak (b) springbok steak (c) kangaroo steak

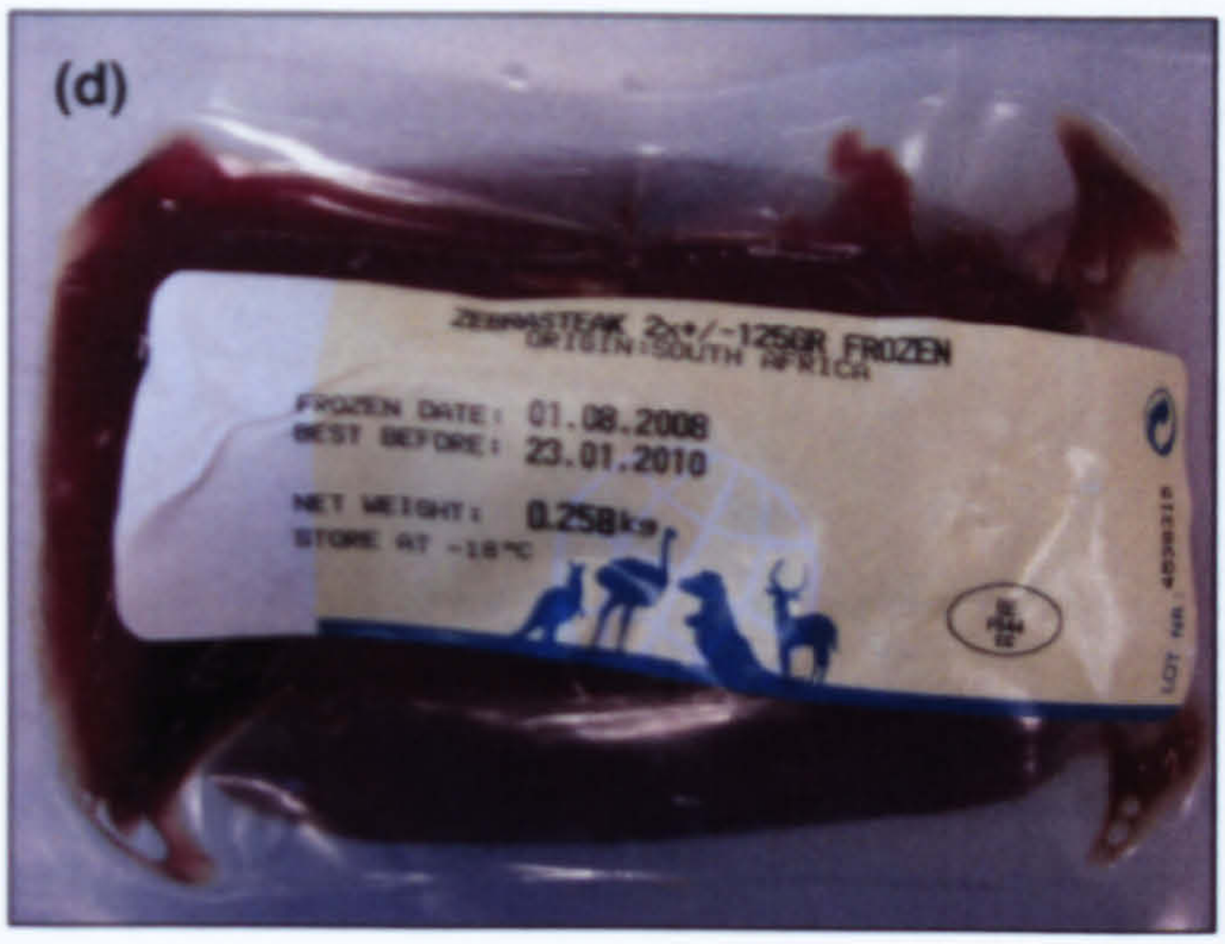

Fig 4.2 cont. Exotic meat products purchased from Company Y (d) zebra steak (b) blesbok steak (f) ostrich steak

Fig 4.2 cont. Exotic meat products purchased from Company Y (g) wildebeest steak (h) bison steak (i) eland steak

Fig 4.2 cont. Exotic meat products purchased from Company Y (j) impala steak (k) camel steak

Company X were: kudu steak, springbok steak, bison burgers and impala burgers. The stated origin of the kudu steak, springbok steak and impala burgers was South Africa, and the origin of the bison burgers was stated as North America. The ingredients listings state that the bison burgers were 95% bison meat; the impala burgers state an impala meat content of 97%. Neither products listed any other animal meat content, and the remaining ingredients were a combination of flavourings, colourings and preservatives. The steak products did not bear ingredients listings and were assumed to be 100% meat from the animal specified.

The products purchased from Company Y were: camel steak, bison steak, blesbok steak, eland steak, impala steak, kangaroo steak, kudu steak, springbok steak, wildebeest steak, zebra steak and ostrich steak. The origins of the products were stated as follows: camel and kangaroo from Australia, bison from the USA, ostrich from the UK and all other products from South Africa. The steak products did not indicate any other ingredients and so were assumed to be 100% meat from the animal stated. A positive control of authenticated domestic horse DNA (*Equus ferus caballus*) was provided by Tepnel Research Products & Services.

DNA Extraction

DNA was extracted from the samples using an Invitrogen™ PureLink™ Genomic DNA Mini Kit following the manufacturers' instructions (Invitrogen Ltd., Paisley, UK). Extraction controls were performed following the same protocol, minus sample. Extracted DNA was quantified using a Nano-Drop® ND-1000 UV-Vis Spectrophotometer (Labtech International, Sussex, UK).

PCR Amplification

Universal primers (mcb398 5'-TACCATGAGGACAAATATCATTCTG-3', mcb869 5'-CCTCCTAGTTTGTTAGGGATTG-3', Verma & Singh 2003) were used to amplify a region of the cyt *b* gene. The primers amplify a fragment c. 420 bp in length (excluding primers) in a wide range of animal species due to their position in a conserved region of the gene (Kocher *et al* 1989), and so are suitable for the analysis of samples of unknown species origin. The cyt *b* gene was selected for analysis in this case rather than cytochrome oxidase I (COI), the gene utilised in barcoding projects (Hebert *et al* 2003a,b), as validated COI sequences are not yet available in the Barcode of Life Data Systems (BOLD) database for many of the (purported) species analysed here. There is considerable sequence data available for cyt *b* in GenBank, with at least one sequence available for all the species expected here. Although the data in GenBank is not validated, the availability of multiple sequences

generated by different projects adds confidence to species matches. Furthermore, 'suspect' matches can be checked by tree-building.

Reaction mixtures (total volume 20µl) contained 1µl of template DNA (at 10-20 ng/ul), 0.36 units ABgene Thermo-Start DNA Polymerase 1.1mM $MgCl_2$, 0.72x reaction buffer (ABgene Ltd., Epsom, UK) and 20 pmol of each primer. Amplifications were carried out using a PTC-200 MJ Research Thermocycler with the following conditions. Initial denaturation at 95°C for 15 minutes; followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 30 seconds; followed by a final extension step at 72°C for 10 minutes. A negative control without template DNA, and a positive control containing 1µl of horse DNA (at 10ng/ul) were run alongside test sample PCRs. Amplification products were visualised under UV light using ethidium bromide stained gel electrophoresis.

Sequencing of PCR Products

Amplified products were cleaned using exonuclease I and shrimp alkaline phosphotase following Werle *et al* (1994). Cycle sequencing of cleaned products was performed using ABI BigDye version 3.1 chemistries (Applied Biosystems, Foster City, CA) using the forward primer, and sequencing products were resolved on an Applied Biosystems (ABI 3730xl) capillary electrophoresis instrument. Sequences were examined and edited using Chromas 2.31 (Technelysium Pty, Tewantin, Queensland, Australia), and compared to sequences deposited in GenBank using a BLASTn search (Altschul *et al* 1990) to obtain species identification. If the species match in GenBank was inconsistent with the product claims, the derived sequence was also compared to a published sequence from the alleged species.

Sub-samples were sent to WDNAS for duplicate analyses to confirm the results of in-house testing.

4.3 Results & Discussion

Visual inspection of the products before testing confirmed that it was virtually impossible to distinguish between some of the meat products by sight alone (Fig 4.1). DNA was recovered from all products, and the cyt *b* gene was successfully amplified by PCR throughout. Different lengths of useable sequence were generated in each case, ranging from 321bp (zebra, Company Y) to 392 bp (bison, Company Y) (See Table 4.1). No DNA was observed in the extraction controls, and no amplification products were observed in the PCR negative controls.

	Stated Species	Stated Country of Origin	Length of Sequence Recovered (b.p.)	GenBank Match Species / Accession Number		Similarity (%)
Company X	Kudu, *Tragelaphus strepsiceros*	South Africa	325	European roe deer, *Capreolus capreolus*	Y14951	99.1
	Springbok, *Antidorcas marsupialis*	South Africa	347	Blesbok, *Damaliscus pygargus*	AF036287	100
	Bison, *Bison bison*	USA	334	* *Bos* spp.*	*	100
	Impala, *Aepyceros melampus*	South Africa	336	Blue wildebeest, *Conochaetes taurinus*	AF016638	99.7
Company Y	Bison, *Bison bison*	USA	392	Bison, *Bison bison*	EU177871	99.7
	Blesbok, *Damaliscus pygargus*	South Africa	330	Fallow deer, *Dama dama*	AJ000022	99.7
	Eland, *Tragelaphus oryx*	South Africa	347	Common eland, *Tragelaphus oryx*	AF036278	98.3
	Impala, *Aepyceros melampus*	South Africa	355	Impala, *Aepyceros melampus*	AF034966	99.7
	Kangaroo, Species not specified	Australia	365	Eastern grey kangaroo, *Macropus giganteus*	EF368023	98.9
	Kudu, *Tragelaphus strepsiceros*	South Africa	362	Kudu, *Tragelaphus strepsiceros*	AF036280	98.3
	Springbok, *Antidorcas marsupialis*	South Africa	387	Springbok, *Antidorcas marsupialis*	AF036281	100
	Wildebeest *Conochaetes taurinus*	South Africa	367	Wildebeest *Conochaetes taurinus*	AF016638	99.5
	Zebra *Equus quagga*	South Africa	321	Impala, *Aepyceros melampus*	AF034966	98.6
	Ostrich, *Struthio camelus*	UK	367	Ostrich, *Struthio camelus*	NC002785	100
	Camel	Australia	387	Dromedary camel, *Camelus dromedarius*	EU159113	99.5

*Multiple 100% matches occurred in GenBank, all for *Bos* species

Table 4.1 Results of the genetic analysis of meat products bought from Company X and Company Y. GenBank matches highlighted in red indicate species matches contrary to product claims.

Comparing the cyt *b* sequences derived from the products to sequences deposited in GenBank indicated that 6 of the 15 samples tested did not conform to the originally indicated material (Table 4.1). Genetic testing performed by WDNAS confirmed the results of in-house testing.

The strength of the GenBank match was not always 100% and ranged from 98.3% to 100% due to single nucleotide substitutions between the query and the sample sequence, which can be attributed to individual variation (Parson *et al* 2000). Such polymorphisms of the cyt *b* gene are common, especially in species with a large geographical distribution (Taberlet *et al* 1992).

Company X Products

The sequence derived from the 'diced kudu steak' most closely matched roe deer sequences in GenBank (Table 4.1), and when compared to a published kudu sequence (Accession no. AF036280) had a similarity of only 82%. This finding, together with the absence of roe deer in South Africa and the location of Company X (near to the Scottish borders) suggests the possibility of UK-end adulteration. Company X also advertises for sale 'Scottish Venison' products, however the website states that this is red deer venison. Given that roe deer is in season all year round in Scotland, whereas kudu has seasonal availability in South Africa, it is feasible that roe deer is being substituted to supply the demand when kudu is not available. Company X did not advertise roe deer products for sale which prevents a direct price comparison, though a nearby company sells roe deer haunch for £0.90/100g compared to the £1.07/100g that the diced kudu steak was retailed for by Company X.

The sequence derived from the 'diced springbok steak' most closely matched *D. pygargus* sequences in GenBank (Table 4.1), and was a poor match to a published springbok sequence (88.9%, Accession no. AF036281). *D. pygargus* is an antelope with two sub-species; the bontebok *D. pygargus pygargus* and the blesbok *D. pygargus phillipsi*. *D. pygargus pygargus* is listed within Appendix II of CITES, whereas *D. pygargus phillipsi* is not CITES-listed. The authors of the sequence in GenBank which gave the highest match do not specify which subspecies the sequence represents. The second closest match was also *D. pygargus* (Accession no. AF016639, match 99.1%), and the authors of this sequence state that this is from the bontebok (Matthee & Robinson 1999). Definitive subspecies assignment was not possible for this sample and further analysis looking at either the control region

(mtDNA) or microsatellites (nDNA) (van der Walt 2002) is required. However, given that the blesbok is one of the primary species on game farms in South Africa (Prins *et al* 2000) and, along with springbok, is the dominant species exported from the country (Hoffman & Wiklund 2006), it seems likely that the sample was blesbok. Company X does not list blesbok products for sale so a direct price comparison between blesbok and springbok could not be made. Interestingly, Company Y sells blesbok fillet steaks for £2.30/100g, whereas Company X sells 'springbok fillet steak' for £4.30/100g.

The 'bison burgers' could not be identified to species level using the amplified fragment, however the species match (100%, multiple species matches) fell within the genus *Bos* – the genus containing the domestic cow – rather than the genus *Bison*. The sample sequence was a poor match (91%) to a published *B. bison* sequence (Accession no. EU177871). The species *Bos gaurus* from south and south-east Asia is often referred to as the Indian bison, which may provide a possible explanation. However Company X's website states that their bison products come from the North American Bison (*Bison bison*). Alternatively, given that the mitochondrial DNA is maternally inherited, the meat could have come from a bovid hybrid. American bison can be crossed with domestic cattle (usually a bison bull is crossed with a domestic cow) to produce animals called 'beefalo' and 'cattalo', which are then often back-crossed to bison or domestic cattle with the aim of producing high protein, low fat, low cholesterol beef. This hybridisation began over 100 years ago (Jones 1907; Polziehn *et al* 1995) which may have resulted in the introgression of domestic cattle genes into the bison populations that contributed to the founding stocks of some contemporary bison herds. Indeed, one study of wild North American Bison found that 5.2% possessed domestic cattle mtDNA (Ward *et al* 1999).

The sequence derived from the 'impala burgers' most closely matched blue wildebeest sequences in GenBank (Table 4.1), and was a poor match to a published impala sequence (85.7%, Accession no. AF034966). Company X sells wildebeest steak, but not wildebeest burgers, so a like-for-like price comparison cannot be made. However Company Y sells wildebeest steak for £2.04/100g, compared to £2.12/100g for impala steak, suggesting that wildebeest has lower commercial value.

No mixed sequences were obtained from the burger products tested which would suggest that meat from only one species was present in each product. For the diced steak samples, only one dice of meat was sampled, so it cannot be stated whether

the product was wholly or partially substituted. However based on this analysis, none of the products tested from Company X appear to be authentic although the bison burgers require further investigation.

Company Y Products

The products tested from Company Y were shown to be authentic, with the exception of the blesbok steaks and the zebra steaks.

The sequence derived from the 'blesbok steaks' most closely matched fallow deer sequences in GenBank (Table 4.1), and was a poor match to a published blesbok sequence (82.1%, Accession no. AF036287). Fallow deer is a European species, and, along with the red deer, is the most common cervid farmed in Europe. The national deer herd numbers in the UK are about 36,000 (Deerfarmer 2003). There is therefore a possibility of UK-end adulteration or mislabelling. However the species has also been successfully introduced into South Africa (Chapman & Chapman 1980), the stated country of product origin, and is now a common feature of South African game farms, so the mislabelling may have occurred before export. To my knowledge, there has been no comprehensive comparison of fallow deer genetic diversity between UK and South African populations, so even further genetic analyses may not be able to provide much more information. Company Y lists fallow deer venison for sale on it's website but does not give an indication of cost or country of origin. Fallow deer steaks are available from another UK company are being sold for £1.50/100g, whereas the 'blesbok steaks' were being sold by Company Y for £2.30/100g.

The sequence derived from the 'zebra steaks' most closely matched impala sequences in GenBank (Table 4.1) and was a poor match to a published *E. quagga* sequence (82.7%, Accession No. FJ785358). Company Y sells 'zebra steaks' for £2.32/100g whereas impala steaks retail for £2.12/100g.

For both Company X and Company Y, what cannot be determined from this investigation is whether or not the adulteration/mislabelling of products occurred in the UK or the source country. Communication with both companies suggests that the products are imported pre-packed and frozen, and that mislabelling would have occurred in the source country. However some of the items bore printed labels (bearing the company's name address, product description etc.) which were presumably added in the UK prior to distribution (see Fig 4.1), opening up the

possibility for mislabelling in the UK. Furthermore, the roe deer result would suggest that some UK-end adulteration had occurred. Wherever the mislabelling took place, and whether or not it was deliberate, this causes serious problems for food traceability. For example, disease transmissions between deer and humans have been reported (e.g. hepatitis E, Takahashi *et al* 2004), and effective control of the disease and dissemination of information to the people who need it can only occur if there is correct species labelling.

It would appear in most cases that the actual species present was of lower commercial value than the species claimed by the product. Falsification of game meat and game-meat products is common due to the profits that result from selling low value meat as meat from more valuable species (Wolf *et al* 1999). However the price comparisons made here can only be used as a guide. Many of the price comparisons were made between companies, which is not ideal, as the different companies may have different suppliers, overheads and pricing structure.

None of the species identified in the products were protected (apart from potentially a bontebok), although there have been other reported incidences of exotic meats being mislabelled to enable the sale of such species. For example, exotic meat dealers in Illinois were found guilty of killing and selling the meat and parts of tigers, leopards, a snow leopard and an Asian swamp deer, all of which are protected under the US Endangered Species Act. The dealers sold the skulls, hides and other parts to local collectors, but sold the meat to a game retailer where it was sold as "lion", which can be legally marketed within the USA (TRAFFIC 2002). This raises the possibility that the UK exotic meat trade could potentially be exploited as a means of importing restricted items. The import of bushmeat (meat from wild animals) for human consumption from West Africa has become a significant problem in recent years (Defra 2007). A survey conducted by WDNAS in 2005-2006 assessing the species identity of meat products confiscated from passengers on African flights arriving at Heathrow airport identified that 3.04% came from 'wild' as opposed to domestic meat, and that 2.17% were from CITES-regulated species (Defra 2007). All potential avenues for the import of bushmeat must be assessed due to the servere consequences of this trade, not only in terms of the conservation risk posed to some of the species involved but also in view of the serious public health risks associated with illegal bushmeat trade (e.g. apes, Peterson 2003). Such assessment will be assisted by the bushmeat barcoding project (see www.barcodingbushmeat.org) and groups such as the Bushmeat Crisis Task Force (see www.bushmeat.org).

Due to growing interest in the importance of food traceability, a number of researchers have developed genetic methods for the species identification of meat and meat products. However many of these are based on mitochondrial DNA (e.g. Matsunaga *et al* 1999) which, as demonstrated in the case of the bison, may not always be suitable due to the occurrence of hybrids. In these cases suitable alternative methods must be employed, for example analysis of nuclear genes, although in the case of bison high levels of nuclear introgression have also been reported (Halbert & Derr 2006).

This study, though preliminary in nature, indicates that a wide-scale assessment of exotic and alternative meats sold within the UK is needed. As well as revealing the true extent of product mislabeling, such a survey would help identify key areas when research into species identification techniques is required.

The results of this study were forwarded to the UK Food Standards Agency, who are currently conducting investigations into the findings.

Chapter 5

General Discussion

Chapter 5: General Discussion

5.1 Introduction

Illegal wildlife trade presents a serious threat to the long-term survival of certain species, which can have serious ecological and socio-economic consequences. By depleting biodiversity, illegal trade undermines efforts to achieve sustainable development and poverty alleviation in regions where people rely on natural resources for their livelihoods (TRAFFIC 2008). Illegal wildlife trade is also linked with disease emergence and transmission (Karesh *et al* 2007), threatens national security through associations with organised criminal and terrorist groups, and results in the considerable loss of human life amongst individuals tasked with protecting targeted species (Wyler & Sheikh 2008).

The work presented here has examined the development and application of molecular tools in the analysis of wildlife products, with a specific emphasis on identifying illegally traded animal parts and derivatives. In this final chapter, the main findings of the work and their implications are discussed in the light of the present status of the field of wildlife forensics and current legislation, and key areas for future work identified.

5.2 Species Identification of Wildlife Products

One of the main barriers to effective enforcement of wildlife trade legislation is the difficulty associated with accurate species identification of wildlife products. Such items are often highly processed, lack diagnostic morphological characteristics, and are associated with high levels of fraud necessitating the application of molecular analyses to establish species origin. A wide range of techniques have been developed and applied to the problem of species identification (discussed in Chapter 1), however these techniques are generally laboratory-based, expensive, and time-consuming. Furthermore, definitive methods of species identification are not yet possible for many products. In cases where the volume of trade is high, routine testing of items may be necessary to ensure effective trade control, though wildlife agencies rarely have the resources to dedicate to such monitoring. There is a conspicuous lack of tools available for law enforcement officers to use for on-the-spot rapid, yet reliable, testing of wildlife products.

In Chapter 2, the development of a field-based assay for the analysis of bear parts and derivatives was described. Although lateral flow immunoassay (LFIA) is a well-established technology, to my knowledge the technique has not been applied to the identification of illegal wildlife products. The system proposed, a cheap, rapid, simple-to-use field test backed up by laboratory-based genetic testing for definitive species identification, could easily be transferred to the analysis of other wildlife products, and would be particularly valuable for high volume trade where fraud is common (e.g. fin and shellfish fisheries, Ogden 2008). Having trialled the bear detection kits, both the London Metropolitan Police (Wildlife Crime Unit) and the CITES enforcement team (Heathrow Airport) gave positive feedback in terms of the test's usability and the rapid delivery of results, and expressed the view that such tools would be invaluable to customs and law enforcement officers working in the field. The conclusion from the WCU was that such kits should be made available to officers in the UK and throughout the EU.

Several specific problems were encountered during the development of the LFIA. For example, inconsistencies in the quality of the membrane initially chosen meant that a new membrane had to be selected and kit development had to be started again from scratch. This inevitably delayed test completion and meant that less time was available to dedicate to the genetic element of the project. Furthermore, the polyclonal anti-bear antibodies were found to cross-react with albumins from a number of different species, and so a considerable amount of time was spent trying to remove this cross-reactivity through affinity chromatography ('scrubbing'). In this case, the cross-reactivity was successfully removed by scrubbing, although this process leads to a considerable reduction in the amount of useable antibody recovered from the goat bleed, and therefore the number of LFIA test strips which can be manufactured from one immunisation. The scrubbing process can also lead to reduced activity in the remaining antibodies due to the number and the strength of the pH changes that occur during the process (Adrian Rogers, Tepnel *Biosystems, pers. comm.)*. An alternative would have been to investigate the use of monoclonal antibodies, which target a single epitope. Although in theory this can make monoclonal antibodies highly specific, if the epitope targeted by the antibody is common to albumins from a number of species, the monoclonal antibody could exhibit similar if not higher levels of cross reactivity than the polyclonal antibodies.

The LFIA is not suitable for the analysis of all bear products available on the market. This is not surprising, given that most LFIAs are optimised to work one sample type

(e.g. home pregnancy-testing kits measure the levels of human chorionic gonadotropin hormone in urine samples). Furthermore although the detection of albumin in bile samples was demonstrated, bile causes a matrix effect which inhibits the binding of bear albumin to the test line to some degree, and, although this could be reduced by the introduction of a sepharose gel separation stage, this compromised the simplicity of the test format and so was not included in the final version of the kit.

Initially, the laboratory-based validation process was intended to be followed by a comprehensive, field-based validation process whereby bear detection kits would be issued to law enforcement officers in a number of different countries. The officers would then use the kits to test suspected bear parts and derivatives and forward the tested items, along with feedback regarding the kits' design and usability, for in-house genetic testing. However, an apparent break down in communication and co-ordination meant that no items were received for genetic testing from these trials. Given the importance of such trials for assessing the kits potential applicability as a law enforcement tool, a separate field trial was arranged with the WCU in the UK, the results of which were included in Chapter 2. Although the results of this trial were invaluable in assessing the kits' user-friendliness, accuracy and for highlighting bear bile products for which more work needs to be done on DNA extraction methods, the sample numbers involved were very small. No genuine bear gallbladders were obtained for testing, so although the LFIA tested negative for the two pig gallbladders tested, it cannot be said conclusively that the LFIA can identify genuine gallbladders. The field trials are, however, ongoing and it is hoped that upon completion, a more comprehensive assessment of the LFIA's performance on various products will be possible.

The in-house assessment of the LFIA was hampered by the fact that the samples available for testing were mostly 'unknowns', in that although a product claims to contain bear parts, due to the high levels of fraud associated with the trade there is a chance that for some of the products the claims could be false. Furthermore, products often did not give an indication of the amount of bear bile present. A product claiming to contain bear bile that subsequently tested negative could therefore be due to a false negative, the amount of bear bile present being below the detection limit of the test or a fraudulent product. Similarly, a product claiming to contain bear bile that subsequently tested positive could either be due to genuine detection of bear albumin, or a fraudulent item producing a false positive. Attempts to detect false

positives included the use of 'bear negative' test strips and the testing, where possible, of like-for-like bear and non-bear products. For example, bear bile wine tested positive, but so did normal white wine, indicating a false positive effect. Such lack of positives is a common problem when trying to develop analysis methods for wildlife products.

Despite these difficulties, the bear LFIA successfully detected bear albumin in a range of sample types and could potentially provide a valuable tool to law enforcers to use for the analysis of bear parts and derivatives. A principal advantage of the test is that of rapid and robust on-site testing. If products are sent for laboratory testing, there is a delay between the seizure of the goods and commencement of any resultant legal proceedings, which can affect the successfulness of prosecutions. Although the system proposed here can only be used to provide reasonable suspicion allowing seizure of goods for laboratory testing, in the future, on-site testing will likely provide definitive species identification, thus eliminating the need for additional laboratory analyses. Such on-site analysis will inevitably be genetically-based. Current developments in nucleic acid detection are guided by two principal trends: miniaturisation of genotyping instruments and high throughput sample analysis (Pereira *et al* 2008), and recent biotechnological advances offer great potential for the development of portable DNA diagnostic devices. Currently, the main limitation of such devices is the requirement of a PCR amplification stage prior to DNA analysis. The development of isothermal strategies of nucleic amplification (e.g. Gill & Ghaemi 2008) may assist the evolution of hand-held DNA diagnostic devices, although improvements in technique sensitivity and assay time must be made before such devices become a reality (Pereira *et al* 2008). In addition, the field of nanobiotechnology offers the potential to analyse DNA without the need for an amplification stage (e.g. Woolley *et al* 2000), enabling high through-put analysis from single DNA molecules.

Until such technology is available, systems such as those proposed in Chapter 2 should be encouraged. Limitations imposed on law enforcement officers regarding cost and expertise required for analysis are circumvented by the cheap, rapid and simple field test, encouraging greater testing of samples. Increased testing will inevitably lead to the identification of more illegal items, and, given that the evidence suggests that increased risks of detection are more of a deterrent to criminals than increased severity of penalties (Ehrlich 1973; Avio & Clark 1978), such systems would likely lead to a reduction in illegal trade.

In parallel to the development of analysis technologies, efforts to identify appropriate markers for species identification and the construction of comprehensive, validated databases must continue. The mitochondrial genes cytochrome *b* (cyt *b*) and cytochrome oxidase subunit I (COI) have been assessed and validated for forensic species identification (e.g. Branicki *et al* 2003; Dawnay *et al* 2007). The principal technique for utilising these markers is DNA nucleotide sequencing followed by comparison of the derived sequence with reference sequences, which may be held in private DNA sequence databases (e.g. Wells *et al* 2001) or obtained from public databases. Matches between query sequences and sequences held in the databases are then used to infer species identification. In the past, public DNA sequence databases have suffered from a lack of quality control and quality assurance, however DNA barcoding projects (Herbert *et al* 2003a,b) are developing public databases specifically for the purpose of cataloguing extant species and for use in species identification. Although not specifically designed for forensic use, such databases offer advantages over traditional public databases in terms of sequence quality and quantity. Indeed the Consortium for the Barcode of Life (CBOL) insists on morphological species identification by independent taxonomists prior to data analysis and provides standard guidelines for data collection and submission, thus reducing erroneous species labelling and standardising inter-laboratory practices. Moreover a vouchering system complements the DNA sequence data base, allowing retrospective checking of putative morphological identity. However as in any large database, errors are inevitable, meaning that even forensically validated databases will contain some erroneous sequences (Salas *et al* 2007). Such inaccuracies do not, however, invalidate the use of such databases for forensic applications, but an understanding of potential error rates and the impact that they have on any inferences made must be fully understood and taken into account.

The primary gene selected for DNA barcoding in animal species is the mtDNA gene COI. However over the course of this thesis, species were analysed for which validated COI sequence information is not yet available in Barcode of Life Data systems database (BOLD). In other public databases (e.g. GenBank) the amount of sequence information available for comparison is greater for cyt *b* than for COI. Although the sequences in GenBank are not validated, the presence of multiple sequences, generated from different projects by different authors, adds confidence to species matches. Furthermore the presence of an unvalidated sequence in GenBank is more informative than no sequence at all in BOLD, and for this reason

cyt *b* was used for species identification throughout this thesis. Future efforts should concentrate on populating the BOLD database with validated COI sequences for forensically important species.

An issue encountered in Chapter 2 was that even when short fragments of mitochondrial DNA are considered, intraspecific variation may be observed. This can result in match results of less than 100% in cases where a species has been insufficiently sampled in the database, which, amongst other issues, can complicate the presentation of results to non-experts (e.g. members of a jury). DNA barcoding projects are attempting to address this issue by aiming to include full species coverage and multiple haplotypes. The inclusion of multiple haplotypes will increase the probability of returning a complete or high percentage match when a query sequence is checked against the database, though increased sequence availability will not necessarily increase forensic utility. For example, as illustrated in Chapter 4, mitochondrial DNA is not always appropriate for species identification (e.g. species identification of 'bison' burgers). A species match must not be treated with complacency, and rather should be interpreted in the light of knowledge of the species in question (it's mating system etc.). The use of nuclear STR markers and assignment tests can circumvent the problem but even then, researchers must assess the possibility of nuclear introgression.

The sample types encountered amongst wildlife products often contain low quantity and/or degraded DNA, which can make it difficult to recover sequences long enough for species identification. Interspecific variation is generally in the form of single nucleotide polymorphisms (SNPs) which can be analysed by non-sequencing approaches, such as PCR-RFLP or SNP genotyping, however due to the reduced genetic information available from such methods they are generally applied to species detection rather than species identification (Ogden *et al* 2009). Such techniques also require the researcher to make prior assumptions about the species present in a sample, though crucially, they enable the detection of species in mixed species samples which is currently a key challenge within the field of wildlife forensics. In this thesis, the power of species identification offered by DNA sequencing was combined with species-specific primers designed using SNP-based approaches (Chapter 2, Peppin *et al* 2008; Chapter 3). In the future, the development of array-based genotyping methods will allow the simultaneous detection of such large numbers of target species within a single assay, that the prior assumption of species in a sample may become irrelevent.

Only a limited amount of genetic work for species identification of bear bile products was possible in the given time frame, however the recovery of DNA from bear bile samples invites the application of a range of different analysis methods. For example, the application of real time PCR and melt-curve analysis should be investigated. As well as providing a potential means of species identification without the need for sequencing, the technique would also enable the absolute quantification of initial template copy number, which was not possible during this study as contamination in the sample extracts interfered with the spectrophotometer readings. Although nuclear DNA was not analysed during this work (preliminary work using microsatellite primers was conducted without success, results not shown), advances in DNA technology may allow the recovery of informative nuclear DNA from bile samples in the future. Combining analysis of DNA recovered from bile samples and gallbladders with population genetic studies could allow the geographic origin of samples to be determined and help demonstrate definitively that an item has crossed an international border, and help to identify and monitor major trade routes of bear products.

5.3 DNA Extraction Methods

Even once suitable markers have been identified for species identification, researchers may still be faced with considerable challenges posed by the recovery of target biomolecules from wildlife products. Certain sample types, for example cooked/processed foodstuffs or manufactured traditional medicines, may be treated in such a way that the molecules of interest are denatured or destroyed. However recovery of useful DNA from challenging sample types has been demonstrated throughout this thesis. Mitochondrial DNA was recovered from bile crystals and processed traditional medicines, and subsequently used for species identification (Chapter 2), and both nuclear and mitochondrial DNA was recovered from rhinoceros horn and subsequently used for species identification and STR profiling (Chapter 4). In both cases commercially available DNA extraction kits were employed with modifications to the manufacturer's protocols. DNA analysis had been deemed unsuitable for the species identification of bile samples by previous researchers; however, improvements in extraction technology coupled with decreases in the sensitivity of analysis methods to inhibitors mean that traditionally difficult sample types can now yield valuable information. Prior to the genetic work detailed in Chapter 2, the methods available for the analysis of bile samples could not provide a

definitive species identification, which was highly problematic given the varying levels of protection afforded to bear species under CITES.

Given the power of DNA analysis for enabling definitive species identification, the wildlife forensics community should make every effort to develop DNA recovery techniques from even the most challenging of sample types. Novel research should be encouraged, as should the transfer and adaptation of technologies from other fields (Ogden *et al* 2009). For example, the kit used at the basis for the recovery of DNA from bile (Chapter 2) was developed for the analysis of human stool samples. Researchers should also seek inspiration from the field of ancient DNA analysis, in which dramatic advances in the analysis of old and highly degraded DNA have been made (e.g. Townson *et al* 1999; Graham 2007). Current developments in the field, such as augmented PCR protocols with an initial DNA-repair step (e.g. Skage & Schander 2007), will also have significant application in the field of forensic research, and increased synergy between the two fields should be encouraged.

As with the validation of the LFIA, a particular problem with the development of extraction methods for items such as processed traditional Asian medicines is that virtually all samples are 'unknowns', in that although a product may claim to contain a particular substance, the claim may be false. If a DNA extraction method fails to yield any results, the researcher is left in some doubt as to whether the extraction technique has failed, or whether the target species DNA was present in the sample in the first place. Some researchers favour 'spiking' sample extracts with DNA of varying concentrations from the target species, amplifying the DNA by PCR and using this to infer whether or not DNA of a certain concentration was present in the original sample (e.g. Wetton *et al* 2002; Yip *et al* 2007). However the DNA that is used for spiking is likely to have been extracted from blood or tissue and therefore be of much higher quality than the DNA that would actually be present in the test product. 'Spiking' may therefore only be useful for indicating the levels of contaminants or PCR inhibitors in the sample extract, rather than whether or not target DNA is present.

The solution here would be to encourage greater co-operation between legitimate traders and the wildlife forensics community. In many cases, the elimination of illegal trade would greatly benefit genuine traders, and given that millions of people around the world rely on wildlife trade for their livelihoods, the preservation of legitimate trade is just as important as the elimination of illegal trade. Traders can provide genuine

samples and in return, the wildlife forensics community can develop techniques for distinguishing legal from illegal products. However, in reality, this is unlikely to work in many situations. For example, the sale of bear gallbladders, bear bile and bear bile products is legal within China, and given the high occurrence of fake items it could be argued that the industry would benefit from the development of techniques to distinguish between genuine and fraudulent items. However reports suggest that the manufacturers of bear bile products benefit significantly from illegal export and sale of the items (Phillips & Wilson 2002), and are therefore unlikely to co-operate in the development of such techniques.

5.4 Individual Identification

The development of individual identification systems (e.g. STR profiling) for forensic application in non-human species has been somewhat limited, and primarily driven by interest in solving high-profile human crimes, such as murder cases. As such, attention has focused primarily on companion animals (e.g. dogs, Halverson & Basten 2005; cats, Butler *et al* 2002), though some studies have also looked at commercially valuable species (e.g. pigs, Putnova *et al* 2003). As wildlife crime is often assigned low priority, STR profiling systems for wildlife species have received less attention. Interest surrounding individual identification as a tool for analysing animal parts and derivatives is, however, growing, due partly to interest in the use of 'genetic registers' to enable the tracking and identification of captive-bred or legally caught individuals, and also due to a desire to link evidence samples (e.g. horn, tusk, blood) to a particular carcass in incidents of wildlife poaching, animal cruelty, or the persecution of protected species (e.g. Dawnay *et al* 2008).

In Chapter 3, mitochondrial genotyping and STR profiling assays were developed for application in the white rhino *Ceratotherium simum*, a species which is protected, commercially valuable and a prime target for poachers. Technical difficulties encountered during the development of the mitochondrial assay meant that it was not possible to assess the levels of variation present in the whole control region, and two fragments of the region were used in the final analysis. The power of identification of the mitochondrial assay was relatively low, and future work should look at assessing portions of the control region not included in this study, including the VNTR region. Due to time and sample constraints only 10 individuals were sequenced for each mitochondrial gene assessed during this study. Future studies should include a more comprehensive survey of mitochondrial variation, to identify additional variable sites which could be incorporated into the assay. The inclusion of more variable sites will

undoubtedly improve the strength of the assay. Furthermore the use of SNP-analysis platforms (e.g. SNaPshot) could be investigated as an alternative to nucleotide sequencing, as this would allow genotyping using much shorter fragments, allowing the analysis of old and degraded samples.

For the STR profiling assay, previously published STR loci were assessed for their suitability for inclusion in a forensic assay. From an initial suite of 16 loci, only 9 were found to be suitable for forensic application. Some of the markers were found to be monomorphic, however given that the loci were reported as polymorphic by their original authors it is possible that the sample size used in this study was too small to detect the variation. In addition, the small sample size meant it was not possible to perform the statistical analyses to accurately determine the match probability generated by the profiling system. Access to a large number of samples held by the South African National Parks (SANParks) authority was secured during the course of the project; however, due to considerable delays, the samples did not arrive in time to be included in the analysis. The small sample size was compromised further by the lack of data accompanying many of the 'wild' samples; the 'wild' samples had been collected previously by staff and students at the University of Pretoria and many were labelled with just the name of the collector without additional location data. The analysis suggested that there was a small but significant difference between the zoo and wild populations (F_{ST} 0.01695). This requires further investigation as if zoos are to be used as effective conservation tools it is important that the captive populations reflect the genetic diversity of wild populations. Future work should include the screening of a much greater number of samples from defined populations to assess the genetic diversity of wild and captive populations, and also to fully assess the forensic utility of the markers. Such screening would also allow the necessary statistical analyses to be performed to determine the strength of the assay for individual identification. Efforts should also be made to identify new STR loci, preferably tri- or tetranucleotide markers which exhibit lower levels of stutter and PCR artefacts. In addition, whole genome sequencing projects should be encouraged to help with the identification of such markers and also assist in the identification of the location of such markers, which is necessary to confirm the independence of loci. In the absence of whole genome sequence information, statistical analyses (linkage analysis) can be used or in previous wildlife studies, the chromosomal location of genetic markers has been investigated using sequence similarity searches between the sequence of the locus in the study species against the whole genome sequence from the most closely related available species, using

an NCBI cross-species megaBLAST (e.g. Dawnay *et al* 2008). However as illustrated in Chapter 3, this is not ideal as even closely related species can have different numbers of chromosomes and so the indicated chromosomal locations may be inaccurate.

Another challenge encountered during the development of the STR profiling system was that the work was carried out in two separate laboratories (Bangor University and University of Pretoria), and instrument-to-instrument variation meant that identical alleles were scored differently in the two laboratories. Sample transfers between laboratories were necessary to calculate correction factors for the two datasets. Further work should look at the development of an allelic ladder to circumvent this problem. An allelic ladder is a sample that produces a profile containing most of the common alleles for each STR marker tested. Profiles generated from unknown samples are compared to the ladder and alleles scored accordingly. An allelic ladder is the only method for providing unequivocal allele assignments regardless of the detection platform employed, and it significantly improves the reliability and reproducibility of the data (Budowle *et al* 2005). The development of allelic ladders is a costly and technically demanding process, which has hampered their development for animal identification systems. Indeed, most wildlife STR profiling systems developed to date lack an allelic ladder, necessitating exhaustive testing between analysis platforms and the development of correction factors is required to allow comparison of data profiles between different laboratories. The development of comprehensive databases for wildlife species would be greatly assisted by the involvement of multiple research groups and increased data-sharing. However, if laboratories are relying on the sharing of samples to calibrate datasets, delays may be inevitable, especially if the species concerned is protected. For example, *C. simum* is a CITES-protected species and the transfer of samples between laboratories was only possible after a lengthy permit application process.

5.5 Conservation Genetics vs. Forensic Casework

The fields of conservation genetics and wildlife forensics are tightly linked. Research methods developed for conservation genetic applications are often modified into valuable investigative tools, and as such the term 'forensic' is increasingly appearing in conservation and population genetics papers. However by their nature, the fields of research ask very different questions: conservation genetics often looks for patterns within populations, forensic casework is concerned with the degree of certainty of identification of a single sample (Ogden *et al* 2009). To this end, Quality Assurance

(QA) and Quality Control (QC) are essential elements of forensic research which help to ensure good laboratory practises, high quality, robust results which will withstand legal scrutiny (Budowle *et al* 2005).

QA and QC are not synonymous; QA refers to the process of determining whether practises and test results are providing reliable information, whereas QC relates to process of verifying whether test conditions are functioning appropriately to yield defined, accurate and reproducible results. Performing validation studies and adhering to laboratory QA and QC increase data confidence for both practitioners and the legal community and are the difference between generating data for conservation genetic research and providing forensic evidence. QA and QC measures are well established in human forensics, largely due to the so-called 'DNA wars' which occurred shortly after forensic DNA typing was first introduced to the legal courts.

In any enforcement investigation, all stages and processes involved in the production and interpretation of data may be challenged. It is therefore essential that all stages of a method are assessed and validated prior to enforcement use. Although validation studies are an essential element of the development of human forensic genetic methods, they are often overlooked in the transfer of conservation genetic research to wildlife forensic application (Ogden *et al* 2009). Furthermore, validation studies on novel techniques and genetic markers are not universally performed or documented by laboratory staff conducting forensic casework on wildlife species, and are often assumed to be expensive and time-consuming (Budowle 2006). However failure to complete such procedures threatens the admissibility of the results as evidence and therefore the success of any associated legal proceedings. An example was seen in the case of Washington v. Tuilefano and Lealuaialii (1998), whereby the defendants were convicted of murder partly due to DNA identification of dog blood and hair recovered from their clothing. An appeal highlighted arguments that the test result should be inadmissible, on the basis that the testing laboratory had not published sufficient data to show that the canine DNA markers and associated probability estimates were reliable (Denver District Attorney 2003). Subsequent to this ruling, a scientific publication identifying the STR loci used for the dog identification in this particular case was published to specifically address the concerns of the Washington court (DeNise *et al* 2004). Other studies of additional canine microsatellite markers have also been published (e.g. Altet *et al* 2001). These

peer-reviewed, scientific publications have been essential in building the integrity of canine-DNA evidence.

At present there are no definitive guidelines for the development of forensic STR profiling systems in wildlife species (but see Dawnay *et al* 2008), though it is recommended that non-human assays be developed according to human DNA forensic guidelines (Budowle *et al* 2005). However, wildlife species present certain challenges which make adhering to human forensic guidelines difficult (discussed in Dawnay 2008; Ogden *et al* 2009), and it is likely that not all recommendations will be met in every case. For example, difficulties in obtaining samples from rare or elusive species may mean that recommended database sizes are not achieved. Furthermore, although individual identification analyses benefit from the transfer of established human forensic statistical methods (Evett & Weir 1998; Balding 2005), important differences exist between human and wildlife systems, in particular with relation to levels of inbreeding (Ayres & Overall 1999; Waits *et al* 2001), necessitating explicit consideration.

The development of formalised recommendations and guidelines for forensic animal identity testing (e.g. Budowle *et al* 2005; Dawnay *et al* 2008b) will aid the adoption of QA and QC practices by those individuals performing wildlife forensic genetic casework, and encourage essential validation studies. As STR profiling systems are developed in progressively more species it is likely that new issues and challenges regarding the development of such systems in wildlife species will arise and need to be dealt with. The guidelines for the development of such systems in wildlife species will therefore evolve over time as knowledge increases.

Standardised and validated procedures are only going to become more pertinent as DNA analysis procedures become increasingly sensitive. With technology promising analysis based on single DNA molecules the threat of contamination is high. If best working practises are adopted now, the wildlife forensic community will be best place to move and grow as these new technologies emerge.

The forensic value of an STR profiling system is tightly linked to the quality of the accompanying population databases. Although guidelines for the validation of STR assays for both human and non-human species have been published, and include recommendations to test for reproducibility, there is little mention of the need to assess error rates within databases as a whole. It was demonstrated in Chapter 3 that STR profiles obtained from different sample types (blood and horn) are subject

to considerably different error rates, and therefore it seems reasonable to recommend that population data be published with an assessment of their accuracy.

5.6 Wildlife Trade Legislation

The Washington Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) is an international treaty which provides a framework for the control and monitoring of wildlife trade, however each of the member countries is responsible for implementing and enforcing CITES within their borders. Consequently, the extent to which legislation is enforced and resources are dedicated to wildlife trade regulation shows major variation between countries. Although it is easy to criticise member countries for a lack of commitment, in reality such enforcement is complex, resource-demanding and, in many cases, beyond the means of many countries concerned. Indeed, in the USA and the UK enforcement systems are well-established, yet illegal wildlife trade still occurs.

Part of the problem is that the legislation is not forensically-led. For example, despite bear gall-bladders from different species being indistinguishable through visual assessment or laboratory analysis (prior to work performed in Chapter 2), some species and populations are listed in Appendix I whereas others are in Appendix II. Without a technique to establish species identification, this legislation is unenforceable, and certainly not worth cash-strapped law enforcement agencies dedicating any time or resources to. At present, the legislation is created and then researchers are expected to play 'catch-up' and develop the necessary techniques to enable the legislation to be enforced. A more sensible approach would be to ask, what are current forensic techniques capable of? It would then be appropriate to develop a legislative framework around the range of potential applications. For example, taking the bear example again, techniques were already available (HPLC, TLC etc.) which could distinguish bear bile and bear gallbladders from the bile and gallbladders of non-bear species. Although these techniques can not provide species-level identification, if the legislation was altered so that trade in bile of gallbladders from *any* bear species was prohibited, a test distinguishing bear from non-bear samples would be sufficient for law enforcement. Other bear parts, which are more easily identifiable (e.g. whole furs) could still be classified under the different appendices.

Law enforcement bodies would benefit from the adoption of uniform legislation across provinces to deal with wildlife trade and related conservation issues. Complex

laws which vary greatly, especially across short geographical distances and species ranges, are not conducive to effective law enforcement. Furthermore the development of new legislation concerning wildlife trade should involve discussion from conservationists, wildlife legislators, law enforcers, members of the legitimate trade community and forensic researchers to ensure that new regulations are relevant, robust and enforceable.

5.7 Focus and Bias of Wildlife Research

Despite a plethora of studies being published on species identification over the last decade or so, they have tended to focus on a few species belonging to three main groups of vertebrates – mammals, actinopterygian fishes and birds, and the number of species for which a method of detection is available is still small (Teletchea 2005). There is also a tendency for researchers to focus on certain, charismatic species as these tend to attract more funding especially from conservation organisations. Thus, research on species identification methods are not always directed towards those species for which development of techniques is most urgent. In response to this, the UK Department for Food, Environment and Rural Affairs (DEFRA) funded a project in early 2000 to develop methods to first prioritise those species at greatest risk from illegal trade, and then to identify where those markers were not currently available but could be of potential use in enforcement cases. This list would then be used by DEFRA to fund researchers to develop DNA markers for those species. The Laboratory of the Government Chemist (LGC) were selected to carry out the project and the final report was published in 2002 (revised 2004, Foy *et al* 2004). Although a system for guiding and advising funding bodies where to direct resources would undoubtedly be useful, the methodology of the report was flawed and could potentially lead to important species being overlooked as research priorities. For example, the report was based around a database that filtered species according to certain criteria, for example CITES/IUCN listing, whether or not molecular markers had been identified in the species, the end result being that species were ranked in terms of research priority order. Using bear species as an example, they were not considered a high research priority given the large number of molecular markers which have been published for this family. However, these markers were developed for ecological and population studies, for use on sample types such as hair and scat, NOT for the analysis of bear parts and derivatives found in trade. Prior to the study detailed in Chapter 2, there were no reports of DNA having been recovered from bear bile samples, and so although many molecular markers had indeed been published for members of the bear family, none of them were useful for the analysis

of bear parts in trade, as there was no published extraction method for such sample types.

The development of a guide for determining where research funds should be directed is undoubtedly useful, though more thought must be given to the selection of the filtering criteria, especially given that for different wildlife products, different key 'questions' will need to be answered by the forensic analysis.

5.8 Summary

Wildlife forensics is a rapidly developing field which can offer solutions to some of the problems encountered by individuals looking to enforce wildlife trade legislation. Over the course of this thesis, the difficulties of species identification and individual identification of specific animal parts and derivatives have been addressed, and methods of analysis developed in response to some of the issues identified. Where possible, techniques have been validated to allow their application to forensic investigation.

Species identification from highly processed and mixed species samples still presents a major challenge to the field, as does the need to develop rapid, robust, field-based methods for the analysis of wildlife products. Interest in the use of 'genetic registers' to track individuals throughout trade, and a desire to link individuals to wildlife crime scenes (e.g. poaching incidents) necessitates the need for reliable robust individual identification systems to be developed for wildlife species. The development of such systems would benefit greatly from a culture of data and information sharing amongst researchers, and also from a concerted effort to develop and implement standardised protocols for technique development, validation and application that can withstand legal scrutiny (Budowle *et al* 2005). Such protocols will prevent non-human evidence being dismissed from court, and therefore help maintain the integrity of the field.

Wildlife trade legislation is fluid and constantly changing to meet economic and conservation needs, therefore the need for novel research and technology transfer is constant. Furthermore, illegal wildlife trade is a global problem, and will require a global, co-ordinated response to be tackled effectively. Efforts should also be made to build wildlife forensic capacity in biodiversity 'hot-spots' which are vulnerable to exploitation and illegal trade, and broad forensic frameworks operating on national and international levels with efficient systems of multi-agency communication should

be developed. Increased co-operation between legitimate traders and the wildlife forensics community should be encouraged, and given the limited resources available to many agencies, effective guidelines should be developed to assist in the identification of priority species for research and technique development.

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Appendix I - Protocols

Appendix 2 – LFIA Validation Results

Appendix 3 – Sequence Images

Appendix 4 – Published Papers

The following material has been excluded from the digitised copy due to 3rd Party Copyright restrictions:

Chapter 2, pages 49 – 126
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Appendices 1 – 4

Readers may consult the original thesis if they wish to see this material.