The application of a PCR based species identification method to African wildlife

By

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Magister Scientiae

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Declaration

I, Lee Darwent, do hereby declare that the research presented in this dissertation was conceived and executed by myself and, apart from the normal guidance from my supervisors, I have received no assistance.

Neither the substance, nor any part of this dissertation, has been submitted in the past or is to be submitted for a degree at this University or any other University

This dissertation is presented in partial fulfillment of the requirements for the degree MSc in Production Animal Studies.

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ABSTRACT

Molecular based species identification is a useful tool in forensic investigations as well as routine work. The ability to cheaply and quickly determine the species of origin of a sample has become increasingly necessary as incidents of wildlife poaching, illegal meat trade and the trade of wildlife products has increased. The current methods of species identification tend to be expensive, time consuming and unreliable.

The use of species-specific primers designed to bind to specific areas in the mitochondrial DNA, has been published. This application has been developed for a small number of domestic animals, however the application of these primers to African wildlife species has yet to be done. This method is relatively simple and is based on specific fragment size amplification using polymerase chain reaction and genotyping.

A total of thirty seven different species were analyzed with this method and 30 of these species were found to have species-specific fragment sizes. A number of different sample types and conditions were tested including uncommon diagnostic samples such as rhinoceros horn, teeth and claws. In addition, the sensitivity of the method was investigated and determined to be very high, detecting species at a DNA concentration of just 0.1 pg/µl.

This method was found to be a highly sensitive, efficient and a fast way to determine species in a number of different sample types and would therefore be of great value in the wildlife trade as these samples can often be of a lower quality or only available in small amounts. The use of this method in forensic science must be done with care due to the problem of cross species amplification. In addition not all of the African Wildlife mammals were available to test, limiting the detection power and specificity of the test.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Species identification has many applications including forensics, speciation and the monitoring of illegal animal and animal product trade (Fajardo, Gonzailez, Rojas, Garcia & Martin 2010). For this reason, it is necessary to have a standard identification system in place. This system would need to efficiently identify a number of different species from various sample types including hair, blood and tissue which are often degraded. The process would need to be fast, highly sensitive, cost effective and allow for high throughput.

The current methods of discerning different species include the use of Short Tandem Repeat (STR) profiling, Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) techniques as well as performing analysis using separate species marker panels, real-time PCR and sequencing (Gupta, Bhagavatula, Thangaraj & Singh 2011; Linacre & Tobe 2011). These methods, however, are often unreliable, time consuming and, in the case of sequencing, very expensive. Another problem often experienced with sequencing is that of mixed samples. If the sample is contaminated by another species' DNA, the results are unreliable as a result of the ability of universal species identification primers to bind many different species. This then requires an additional multiplex PCR to be performed. Some of the nuclear STR markers used are not species-specific and this is a problem with mixed samples.

The current method of species identification in forensic genetics laboratories differ, however a common factor is that identification requires a number of tests and this is both time consuming and expensive. A cost effective and quick species identification method using a single set of primers, PCR and capillary electrophoresis would be an advantage. Such a method has been published for 18 mammals and four birds, however, this method has not been

applied to African wildlife species. In this study this method is applied to a variety of African wildlife species.

1.2 The use of species identification commercially and in Forensic Science

The use of genetic tests for species identification is becoming more popular for commercial and forensic use (El-Sayed, Mohamed, Ashrm & El-Rahman 2009). The growing popularity of genetic testing can be attributed to the reliability of the results, the specificity that can be attained and for forensic purposes the strong evidence that can be presented in court cases.

Wildlife crime is a growing problem in a number of countries including South Africa. It includes live animal trafficking and illegal poaching for both meat and trophies. Fraudulent mislabeling of game meat products is increasing due to the large profit margin and monitoring this is a difficult process (Fajardo *et al.* 2010). Another market which has increased the rate of wildlife crime is the use of animal parts such as rhinoceros horns and elephant tusks in the making of traditional medicines, jewellery and ornaments (Greg 2004; Humphreys & Smith 2011). An example of this is the large increase in rhinoceros poaching in South Africa since 2009 (African Rhino Specialist Group; http://www.rhinos-irf.org/afrsg). The number of both white and black rhinoceros being poached has dramatically risen due to the demand in Asian markets and this has led to renewed interest in the use of genetics for species identification. A similar situation has occurred with tigers with the World Wildlife Fund reporting that only 3200 exist in the wild (http://www.worldwildlife.org/species/finder/tigers/publications.html). In both cases it is body parts of the animals such as powdered rhinoceros horn, tiger claws or bone that are traded rather than the whole animal and this can lead to problems in species identification, especially if only a small piece is traded. In suspected poaching or illegal wildlife trade cases an efficient and reliable method of species identification can be a vital tool in determining whether a

wildlife crime has been committed and if it has, in the subsequent prosecution of the offender.

1.3 Current methods of species identification

The simplest methods of species identification are initial morphology or microscopy. These methods are, however, not always an option as the sample may be degraded or could be traded in a different form from the original. In addition, if microscopy is used to examine, for example, a hair sample, a highly specialized technician is needed to do the comparison and there is room for personal interpretation which can lead to individual bias. In cases where the results are to be used in the prosecution of an individual, this potential bias can introduce doubt in the evidence. In these circumstances the samples are often sent for molecular testing. The most common molecular method of species identification is the sequencing of certain genes mostly from the mitochondrial genome (Caine, Lima, Pontes, Abrantes, Pereira & Pinheiro 2006; Murugaigh, Noor, Mastakim, Billing, Selemat &Radu 2009; Prado, Calo-Maya, Villa, Cepeda & Barros-Velazquez 2007) as well as the 12S and 16S rRNA loci (Kitano, Umetsu, Tian & Osawa 2007). This, however, is a time consuming and expensive method. In addition, highly degraded samples may not generate sufficient data to use for the identification and may produce mixed sequences as a result of a sample containing more than one sequence. A simpler and less expensive method is required.

The traditional molecular methods for species identification include the use of protein precipitation and electrophoresis, however, these methods have been found to be time-consuming and inaccurate (El-Sayed *et al.* 2009). Other common immunological methods such as HPLC analysis of haemoglobin or fatty acid discrimination have been used, however, they have some problems (Parson, Pegorapo, Niederstatter, Foger & Steinlechner 2000), including the limited species with corresponding antibodies for these tests and the costly maintenance of representative antigen set (Parson *et al.* 2000). Lack of specificity at the species level, where only the family can be identified, and

cross reactions are further problems. Immunological methods are dependent on the use of the sample proteins (Parson *et al.* 2000), and if the sample has suffered unfavourable environmental conditions, it is not possible to use these methods. This is due to the denaturation of the soluble proteins during food processing or heat-treatment (Fajardo *et al.* 2010).

A DNA-based method of species identification uses random amplification of polymorphic DNA (RAPD) (Lee & Chang 1994; Martinez & Malmheden Yman 1998). This method allows a certain "fingerprint" to be created for each animal. The main problem with this method is the fact that degraded samples cannot be used (Martinez & Malmheden Yman 1998) and this is often a major limitation with forensic evidence samples. As noted by Martinez & Malmheden Yman 1998, a problem can arise with results of RAPD analysis when DNA from more than one different species is mixed. Another disadvantage of this method is the lack of reproducibility. In order to obtain reproducible results, the PCR conditions need to be strictly standardized between laboratories and this is unrealistic. This process is time consuming, expensive and has now been replaced with the use of Restriction Fragment Length Polymorphisms (RFLPs).

A common method used for the identification of animal products such as milk and meat, is the use of PCR-RFLP's and short segment repeats (SSR). These have been successful in distinguishing between different animal products. Abdel-Rahman *et al* (2007) used a PCR and PCR-RFLP technique to differentiate between buffalo, cattle and sheep milk. This is important to maintain industry standards and fairness. While an initial PCR was able to distinguish sheep from the other samples, extra work was necessary to distinguish between cattle and buffalo milk (Adbel-Rahman *et al.* 2007). This method, while expensive and labour intensive, is currently the most widely used. PCR-RFLP analysis has been performed on samples such as dog, badger, cattle, human and pigs, among others (Adbel-Rahman *et al.* 2007; Bataille, Crainic, Leterreux, Durigon & de Mazancourt 1999; Bravi, Liron,

Mirol, Ripoli, Peral-Garcia & Giovambattista 2004; El-Sayed *et al.* 2009; Prado *et al.* 2007)

Another method commonly used for the identification of species is the use of short tandem repeat (STR) profiling systems (Angleby & Savolainen 2005 ; Dawnay, Ogden, Thorpe, Pope, Dawson & McEwing 2008; Eichmann, Berger, Steinlechner & Parson 2005). This involves the use of genetic markers and is more suited to identification at the individual level. STR profiling systems work using statistical probabilities that two evidence samples are the same (Dawnay *et al.* 2008). It has been reported that STR profiles for wild animals are available however the data is limited and species specificity has not been validated for all the wild species. There are no guidelines that outline the number of STR loci that produce valid results in terms of the statistics in wild animals and this introduces room for differing interpretation. Haunshi *et al.* (2009) developed species-specific primer sets that amplified markers that successfully identified chicken, duck, pigeon and pig meat. While they were successful, the larger number of primers used, increased the cost of the test, decreasing its economical viability.

Pyrosequencing is based on the detection of pyrophosphate (PPi) that is released from dNTPs during DNA synthesis. As this happens visible light is generated and this is directly related to the number of nucleotides being incorporated. The 12S and 18S regions of rRNA have been used in designing a species-specific DNA pyrosequencing method (Karlsson & Holmlund 2007). While this was successful in differentiating between 28 different mammals, it is expensive and requires specialized equipment.

Tobe *et al.* (2008) described a test in which species-specific primers were developed for a number of species using the cytochrome *b* gene in the mitochondria. An area of high homology was found and from this three universal forward primers were developed. Two species-specific reverse primers for each species were designed to react with one of the three labelled universal forward primers. These amplified fragments in all the species used

in the study (Tobe & Linacre 2008b; Tobe *et al.* 2008c). They were designed to bind with only the species for which they were designed. This method of species identification was successful in differentiating between different species, using a number of different reverse primers.

Nakamura *et al.* (2009) designed an efficient and quick method of species identification using multiplex PCR and capillary electrophoresis. Three sets of primers were developed for the amplification of the mitochondrial DNA hypervariable region in mammals (including most domestic animals such as horse, cat and dog), birds and fish. Each of these primer sets were fluorescently labelled with FAM™, NED™ and VIC® labels which corresponded to the products of mammals, birds and fish respectively. These primers were then used in a multiplex-PCR and amplicons analysed on a genetic analyzer using capillary electrophoresis (Nakamura *et al* 2009). The results produced fragment peaks of specific sizes which corresponded to different species. Once validated these fragment sizes (shown as peaks) were used to set up a library from which one could look at the fragment obtained and identify the species without sequencing being required. These species-specific fragments did differ slightly in length due to insertions/deletions in the sequences of certain species therefore a threshold value of approximately 2-3 bp was determined for the different species (Nakamura *et al.* 2009). Some overlap between species did occur between human and bovine where the same size fragment was observed. This led to another human primer set being developed in order to differentiate between the two species. This method is both time efficient, cost effective and can differentiate mixed samples efficiently making it an ideal system to use for species identification. These primers have however not yet been applied to wildlife species.

1.4 Mitochondrial DNA (mtDNA)

Mitochondrial DNA (mtDNA) has been widely used in the field of population genetics and phylogenetic studies (Bataille *et al.* 1999; Nakaki, Hino, Miyoshi, Nakayama, Moriyoshi, Morikawa & Itohara 2007; Pereira, Meirinhos, Amorim & Pereira 2006). Mitochondrial DNA has also played an important role in a number of forensic investigations in both the human and animal forensic fields (Matsuda, Seo, Kakizaki, Kozawa, Muraoka & Yukawa 2005; Nelson & Melton 2007; Rastogi, Dharne, Walujkar, Kumara, Patole & Shouche 2007).

There are a great number of reasons and advantages for using mtDNA as opposed to nuclear DNA. Firstly mtDNA can be found in samples that do not contain much nuclear DNA or in which the DNA is degraded (e.g. hair, skeletal tissue and degraded samples). This is important when dealing with cases in which evidentiary material is limited (Nelson *et al.* 2007). Mitochondrial DNA is hundreds to thousands of times more abundant than genomic DNA (Bellis, Ashton, Freney, Blair & Griffith 2003; Prado *et al.* 2007). This is because there are about 800-1000 mitochondria per cell and each mitochondrion contains 2-4 mtDNA molecules. mtDNA is therefore a naturally amplifiable source of genetic material and therefore a valuable resource in forensics (Zha, Xing & Yang 2011). A study published by Andreasson *et al.* (2006) used real-time PCR to determine the nuclear and mtDNA concentrations in different forensic samples. They found that mtDNA levels were substantially higher in all the samples including shed head hair and plucked hair. In addition mtDNA is packaged and protected in its own organelle (mitochondrion) and thus is better preserved in degraded samples. Due to the high copy number of mtDNA, it evolves at a much faster rate than nuclear DNA (Murugaigh *et al.* 2009). This causes mtDNA to be highly variable (Prado *et al.* 2007). This is also due to the inefficiency of the DNA repair mechanism (Clayton 2004). Different regions of mtDNA also evolve at different mutational rates. mtDNA codes for functional proteins and does not undergo recombination and therefore it cannot mutate unconditionally. This adds variability to the DNA while a certain level of conservation is maintained.

A major limitation of working with mtDNA is the fact that it is maternally inherited and therefore represents only the maternal lineage of the animal (Rastogi *et al.* 2007). This could lead to problems when looking at hybrid species. As most of the variation in species such as these will be found in the nuclear DNA rather than the mtDNA, there could be a lack of distinction between an original species and a hybrid individual. In such a case primers based on mtDNA would not be able to detect differences between the hybrid species and the original female species. In addition heteroplasmy in mtDNA has been reported and this will further complicate the results (Bellis *et al.* 2003; Xiufeng & Arnason 1994). In the case of heteroplasmy, one individual animal can have two different copies of mtDNA due to a novel mutation or a mutation that has been inherited. However this should only be an issue when looking within species and should not affect between-species identification.

1.4.1 Cytochrome *b* and cytochrome oxidase 1

There are a number of mitochondrial genes or regions which can be used for species identification and these include cytochrome *b*, cytochrome oxidase 1 (CO1), 12s and 16s rRNA and the hypervariable or control region (mtDNA-HV) found within the D-loop region of the mitochondrion (Figure 1).

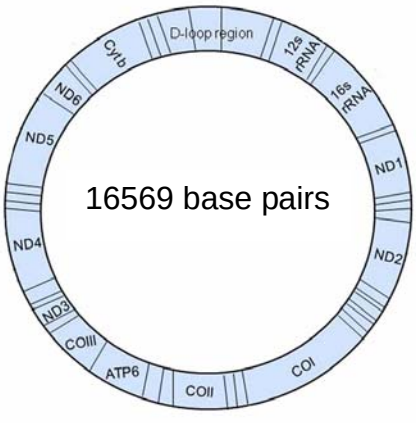

Figure 1: The mammalian mitochondrial genome. The gene order is the same in all mammalian species. Figure adapted from Tobe and Linacre, 2010

Of these candidates, the most commonly used in species identification is cytochrome *b* as it is species-specific (Hsieh, Chiang, Tsai, Lai, Huang, Linacre & Lee 2001; Linacre & Tobe 2011; Matsuda *et al.* 2005; Parson *et al.* 2000) and is therefore an ideal target for identification.

Cytochrome *b* is involved in electron transport in the respiratory pathway of mitochondria. It contains eight transmembrane helices connected by intramembrane or extramembrane domains (Hsieh *et al.* 2001; Linacre *et al.* 2011). It is the only cytochrome coded by mtDNA (Carodoso, Gonzalez-Fernandez, Odriozola, Valverde & de Pancorbo 2008). A 385bp region of cytochrome *b* has the widest taxonomic representation in nucleotide databases with over 8000 cytochrome *b* gene sequences for vertebrates available in GenBank (Irwin, Kocher & Wilson 1991).

The cytochrome *b* gene is the most widely used gene for phylogenetic work for a number of reasons. Although it evolves slowly in terms of non-synonymous substitutions, the rate of evolution in silent positions is relatively fast (Irwin *et al.* 1991). Cytochrome *b* has both variable and conserved regions with variable regions not subjected to functional constraints. Most of the variable positions seem to be located within the coding regions for transmembrane domains or for the amino and carboxy-terminal ends (Irwin *et al.* 1991).

Cytochrome *b* has been the common model gene for population genetics, however in the case of species identification, cytochrome oxidase 1 (CO1) has been more widely supported (Tobe, Kitcherner & Linacre 2009). Cytochrome Oxidase 1 which was initially used in the identification of invertebrate `species has been proposed as the locus for a barcode of all animal species (www.barcodeoflife.org). This use of CO1 in species identification is based on the sequencing of the CO1 gene in the sample and the comparison of these results to known sequences published on Consortium for the Barcode of Life (CBOL). This leads to a reliance on CBOL and the test is limited to the species that have been uploaded onto CBOL. This also

creates a problem with cases when DNA concentration and quality in a sample is very low and the sequencing results can only give partial sequences. In the event of a mixed sample, one species concentration could be significantly higher than another and this could mask the appearance of the species in lower concentration. Despite this fact, researchers in South Africa have used this method of COI sequencing successfully in three forensic wildlife cases (Dalton & Kotze 2011).

The debate about which gene is more applicable has been fought for many years, however cytochrome *b* was found to offer greater informative value when looking at a relatively smaller fragment (Tobe *et al.* 2009). In recent studies looking at meat authentication, a 358bp cytochrome *b* amplicon was produced from 44 different animal species covering the 5 major vertebrate groups. Restriction analysis was done and successfully differentiated between the different species (Parson *et al.* 2000). This indicates that there is enough variation within the cytochrome *b* gene for species-specific primers to be designed. Similarly the same 358bp fragment was used by Bravi *et al.* (2004) to identify canine samples from a number of contaminating species. They used the PCR-RFLP method and found there was enough sequence divergence to distinguish between species. The previous studies using cytochrome *b* indicate that there were enough areas of variability for the primers to distinguish between different species. While both cytochrome *b* and CO1 showed a large amount of sequence diversity, the reliance on sequencing or other methods such as RFLP's increase cost and time required for species identification. In all the previous studies using cytochrome *b,* a number of primer sets were used and this again increased the cost and complexity of a species identification test.

1.4.2 mtDNA Hypervariable region

Another region of great interest is the mtDNA hypervariable region (mtDNA-HV). This is found within the D-loop of mitochondrial DNA (Figure 1). This is located in the mtDNA control region which is an area of the mitochondrial

genome which is non-coding. The displacement loop or D-loop is a DNA structure where two strands of a double stranded DNA molecule are separated and held apart by a third strand of DNA. The function of the D-loop is not yet clear, but research has suggested that it participates in the organization of the mitochondrial nucleoid. Certain bases within the D-loop region are conserved, but there are areas that are highly variable and these are called the hypervariable regions.

This locus has been used less in species identification but has rather been used in individual or intraspecific determination. This is due to its higher level of variability (nucleotide diversity is this region is on average 1.7%) in certain regions and sequencing of this region is popular. Kocher *et al.* (1989) described universal primers that were designed to amplify the D-loop of mtDNA, however the interspecific variability was low and therefore this test did not provide an efficient species identification method (Kocher, Thomas, Meyer, Edwards, Paabo, Villablanca & Wilson 1989). In addition, the mtDNA-HV region contains regions with high conservation including the flanking regions and these can be exploited. Nakamura *et al.* (2009) used these highly conserved regions and designed universal primers mt-U1 and mt-U2 which hybridize to these regions. These primers allow for the amplification of species-specific fragments of a certain size (in base pairs) which can be visualized using capillary electrophoresis and compared to a control sample. In certain species more than one fragment was amplified. This was due to the variable number of tandem repeats in these species mtDNA-HV which led to a length heteroplasmy. This was noted in the control sample therefore allowing for comparisons and species determination. In the case of the *Homo sapiens* mtDNA-HV region, the fragment amplified was equal in size to that of *Bos indicus*. This led to the design of two extra primers which were able to further differentiate between the two species. In addition these fragments could be sequenced although this was not necessary in every case. The mtDNA-HV region has been shown to be a successful locus from which to design primers that will effectively differentiate between species.

1.5 Molecular Methodologies

1.5.1 DNA Extraction

There are a number of different extraction methods for the different sample types including the use of commercial kits. The disadvantage of using a kit for routine sample extractions is that this will increase the cost of processing a large number of samples. The use of the commercial kits, however, is useful for non-routine samples as the quality of DNA extracted is potentially better and these kits contain specific protocols for extraction from the more uncommon sample types. The standard laboratory extraction method used in the Veterinary Genetics Laboratory (VGL) for tissue, blood and salted tissue extraction employs the use of PCIA which facilitates the removal of unwanted proteins from the extraction by separating these proteins into a separate layer based on density (Sambrook, 1989), which can then be discarded. The extraction method for hair samples uses alternating alkaline and acidic solutions in order to break open the hair follicles to allow access to the DNA within. These methods are standard laboratory procedure and allow for efficient extraction of both mtDNA and nuclear DNA. This is important as a sample need only be extracted once and can be used in processes requiring either mtDNA or nuclear DNA.

The different DNA extraction methods used for the different sample types (e.g. hair extraction vs. blood extraction) yield extracts with differing concentrations and quality levels. DNA molecules absorb ultraviolet light and using a spectrophotometer, a sample is exposed to ultraviolet light at 260 nm, and a photo-detector measures the light that passes through the sample. The more light absorbed by the sample, the higher the nucleic acid concentration in the sample. The instrument used for this procedure was the Nanodrop ND-1000 (NanoDrop Technologies).

In addition nucleic acid samples can be contaminated with other molecules such as sample proteins not removed efficiently during the extraction

procedure. The ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) is used to assess the purity of DNA. The optimal range for this measurement is 1.8 – 2. http://www.iscpubs.com/Media/PublishingTitles/b0608kar.pdf

1.5.2 Polymerase Chain Reaction

PCR is a method used to amplify a targeted region of a DNA sequence, producing thousands of identical copies. The method was developed in 1983 by Kary Mullis and consists of cycles of repeated heating and cooling of the reaction which allows for the enzymatic replication of the DNA (Mullis & Faloona 1987). A PCR requires the use of a number of reagents all allowing for amplification of DNA.

Firstly the DNA template is necessary from which the target region is to be amplified. Primers are then designed that are complementary to the 3'ends of each of the sense and anti-sense strands of the DNA target. These allow for the selection of a specific region of the DNA to be amplified. Deoxynucleotide triphosphates (dNTPs) are added to the reaction as these are used by DNA polymerase to synthesize the new DNA strand. $MgCl_2$ is added to the reaction in order to aid the binding of the primer to the template DNA by complexing with the single-stranded DNA. A buffer solution is included in the reaction as this allows for the correct environment for the activity of the DNA polymerase (Sambrook 2001)

The first step in the PCR is the heating of the reaction to the temperature required to activate the DNA polymerase. This is only necessary for hot-start PCR. Hot-start PCR is advantageous as it decreases the chances of mis-priming, non-specific primer annealing and primer dimerization (Sharkey, Scalice, Christy, Atwood & Daiss 1994). The next step is the denaturation of the DNA template. By heating the reaction, the hydrogen bonds between the complementary bases in the DNA molecule are broken and the two DNA strands separate. Next the temperature is lowered and this allows for primer annealing to the template DNA. The Taq polymerase then binds to the primer-template molecule and DNA synthesis begins. The temperature is then raised

to the optimal activity temperature of Taq polymerase and a new DNA strand complementary to the template strand is synthesized (extension step). dNTPs are added in the 5' to 3' direction and the time taken for this is dependent on the length of the DNA fragment to be amplified. These steps are then repeated for a determined number of cycles. The final elongation step is performed in order to ensure that any single-stranded DNA is fully extended. The reaction temperature is brought down to 4ºC at which point the reaction is stable (Innis, Gelfand, Snisky & White 1990).

Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences (Veenstra-Vanderweele, Hanna, Leventhal & Cook Jr 1998). This reduces the time taken to process many samples and the volume of reagents used. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and the amplicon sizes need to be different enough to form distinct bands when visualized (Veenstra-Vanderweele *et al.* 1998). Fortunately capillary electrophoresis is able to distinguish sequences with a single base pair difference and allows similar sized fragments to be labelled with different dyes so this is less of a problem (Budowle 2000).

1.5.3 Capillary electrophoresis

Capillary electrophoresis is a technique that allows for the real-time fluorescent detection of differently sized DNA fragments (Budowle 2000). It also eliminates manual gel electrophoresis and is more specific (differences of 1 base pair can be identified (Veenstra-Vanderweele *et al.* 1998). Capillary electrophoresis consists of fused silica capillary between two electrolyte reservoirs. By running an electric field between these reservoirs, the differently sized fragments can be separated and detected (Budowle 2000). Fluorescent tags can be bound to the 5' end of primers included to aid in the detection of the different fragments (Veenstra-Vanderweele *et al.* 1998) and this mode of detection offers higher sensitivity. The different fragments are

then detected by a laser through the capillary wall. The data is captured and analyzed using software such as STRand, which has been developed and used at the University of California, Davis' Veterinary Genetics Lab (http://www.vgl.ucdavis.edu/STRand)

It automates the analysis of DNA fragment length polymorphism samples run on fluorescence based capillaries. The program uses the FSA file format which is produced from the ABI 3130xl Genetic Analyzer. STRand converts the results into a electropherogram which shows the size of the fragment (sample migration) versus the fluorescence (level of amplification) (Grossman & Colburn 1992).

1.5.4 Sequencing

Sequencing of DNA allows one to determine the exact sequence of nucleotides in a DNA molecule. These sequences can then be compared to sequences found on the database GenBank. The first DNA sequences were obtained in the 1970's using laborious methods, however in 1977, Frederick Sanger developed the Sanger Dideoxy sequencing method (Sanger & Coulson 1975). During PCR amplification of the sample, fluorescently labelled dideoxynucleotide triphosphates (ddNTPs) are added to the reaction. These molecules lack hydroxyl groups, therefore when the DNA polymerase adds the nucleotides to a growing chain, if a ddNTP is added, no further nucleotides can be added. This causes different sized fragments, which end in different bases, to be synthesized. These fragments are then separated by capillary electrophoresis and passed through a laser beam which detects the fluorescence if each ddNTP (Smith, Sanders, Kaiser, Hughes, Dodd, Connell, Heiner, Kent & Hood 1986). A computer software program then interprets the fluorescence of the ddNTPs and constructs electropherograms from this information. These are then used to read the sequence of the DNA molecule based on the different coloured peaks, using Sequencing Analysis 2.5 (Applied Biosystems Sequencing Analysis 5.2.0). The primer set mcb 398 and mcb395 designed by (Verma & Singh 2003a), is commonly used in sequencing unknown samples and is designed to amplify a specific region of

the cytochrome *b* gene. This is an ideal region to target as it is widely represented on GenBank.

1.6 Research questions and objectives

Will the application of a previously published, mitochondrial DNA based species identification method, using PCR and capillary electrophoresis successfully identify and distinguish a number of African wildlife species?

The objectives of this study were as follows:

- To apply the species identification system designed by Nakamura *et al.* (2009) to a variety of selected African wildlife including black and white rhinoceros, wild felid species and a variety of antelope species.
- To validate the test in terms of DNA concentration and sensitivity.
- To identify the fragment obtained by sequencing and BLAST analysis.
- To validate the test using known samples and known sample mixtures.
- To create a reference library from which different wildlife species can be identified on the basis of the fragment amplified.

1.7 Delineations and limitations

1.7.1. Limited access to all the morphologically identified representative species of African wildlife to act as controls.

1.7.2. Species with overlapping fragments that cannot be distinguished using this method.

1.7.3. Species in which the test fails as a result of primer binding site problems.

1.7.4. The interference of the fluorescence of the dye labelled primers in the sequencing of the fragments.

1.7.5. The amplification of nuclear pseudogenes of the mtDNA could produce confusing results.

1.7.6. Number of samples required to validate the test and address the issue of individual variation using this mtDNA region.

1.8 Abbreviations

PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
PCIA	Phenol-Chlorophorm Iso-Amyl Alcohol
RAPD	Random Amplified Polymorphic DNA
mtDNA	Mitochondrial DNA
HV	Hypervariable Region
CR	Control Region
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
BLAST	Basic Local Alignment Search Tool
bp	base pair

CHAPTER 2: METHODS AND MATERIALS

2.1. Sample Selection

Samples from the VGL sample collection at Onderstepoort, University of Pretoria and a taxidermist were used. This was important as the taxidermist was able to morphologically identify the samples and therefore the control species were attained from a credible source. Initially the samples chosen were those of domestic species in order to determine if the primers were working and to optimize the PCR process. The samples used in this initial setup stage were all tissue samples.

Once the PCR was optimized, different species and sample types were tested. Specifically blood, hair, skin, organ tissue, and other sample types from VGL and salted skin from a taxidermist were tested. This was done to test the sensitivity of the test to different sample types as well as what the limitations of the test were. It was decided to focus on African mammal species and a list of these species was created. In addition, species commonly involved in illegal animal product trade, such as tigers, were included if a positively identified sample was available. Obtaining samples for all these species was not possible, so the method was tested on the available species. In particular the obtaining of different avian samples was difficult as the VGL does not routinely receive a large number of avian species. For each species at least two samples from different animals were tested. In addition both a domestic animal mix and a wildlife animal mix were made. Within these mixtures different DNA concentrations of different species were present. This was done to test the efficacy of the test in picking up multiple species within a sample, as well as whether DNA concentration would play a role in the sensitivity of the test.

2.2. DNA Extraction

A number of different sample types were used including blood, hair and tissue. DNA extraction from hair samples requires that the DNA in the cell nucleus be reached by the breaking of the hair follicle. Six hair roots were incubated at 97ºC for 15 minutes in 200mM sodium hydroxide (NaOH; Sigma-Aldrich, St. Louis; MO) in the first alkaline lysis step. An acidic buffer consisting of 200mM hydrochloric acid (HCl; Saarchem, MERCK, Midrand; Gauteng) and 100mM Tris-HCl, pH8.5 (Tris base; Promega, Madison; WI) was then used to neutralize the solution before it was used for PCR.

DNA was extracted from 500µl un-clotted blood which required an initial wash step using 10mM sodium chloride (NaCl) (Promega, Madison; WI) and 10mM Ethylene Diamine Tetra Acetic Acid (EDTA, pH 7; Promega, Madison; WI). This step lysed the red blood cells which then remain in solution after centrifugation. This supernatant was then discarded leaving behind a pellet consisting of white blood cells and proteins. The pellet was then incubated for two hours at 56ºC in a solution of 10mM Tris-HCl (pH 8), 10mM EDTA, 50mM NaCl, 20% sodium dodecyl sulphate (SDS; BDH Laboratory Supplies, Poole; England) and 20 mM/ml proteinase-K (Sigma-Aldrich, St. Louis; MO). This buffering solution broke down the proteins in the pellet as well as lysing the white blood cells to release the DNA from the cell nucleus. The samples were then purified using the phenol-chloroform-isoamyalcohol (PCIA) method. Phenol-choloform (PCIA; Sigma-Aldrich, St. Louis; MO) purified the samples by degrading the contaminating proteins and separating the organic phase from the aqueous phase (Sambrook, 1989). The DNA was then precipitated and washed using ethanol (EtOH) and resuspended in Tris-EDTA (TE; Promega, Madison, WI).

The method for extraction of tissue samples was similar to the extraction of the blood sample with the only difference being the exclusion of the initial wash step as this was unnecessary. The tissues were cut into small

(2mmx2mm) pieces and the extraction method was followed from the incubation step.

For the salted tissue samples from the taxidermist, the cut up samples were initially rehydrated in ddH_20 overnight, with the water being changed twice during a 12 hour period. Following this the samples were treated as normal tissue samples.

2.3. Sample quality and concentration

The concentration and quality of the DNA extracts had to be monitored and optimized for the test. To obtain this, a spectrophotometer (Nanodrop ND-1000 ®, Nanodrop Technologies) was used to quantify the extracts. The DNA concentration initially used was around 10 ng/µl. Following this, a dilution series was done on these samples in which the DNA concentration was decreased from 10 ng/µl to 1 pg/µl using water in order to dilute the DNA levels in the samples. The different concentrations were tested in order to determine the ideal DNA concentration of the sample for the test. Once the ideal DNA concentration was determined, the samples were used at this concentration.

2.4. Primer selection

The primers described in Nakamura *et al.* (2009) were used in this study and the sequences are shown in Tables 1.1 and 1.2. Eight primers were used, of which four were fluorescently labelled. The primers used were the mammal-specific primer set which consisted of the universal forward and reverse primers (6FAM-mt-U1F and mtU2R) and in conjunction human-specific primers (6FAM-mt-HV2F and mt-HVR). Four bird primers were used and these were avian specific primers, which could be used if the sample was suspected to be of avian origin (mt-Bd6F, mt-Bd6R, mt-Bd7F and mt-Bd7R).

The addition of an M13 sequence was added to each primer pair in order to potentially assist with the sequencing of the fragments.

Table 1.1: Sequences including M13 tails (underlined) and dye labels of the mammalian primer set and human specific primer set

Name	Sequence (5' to 3')	Primer length (bp)	GC%	T_m (°C)
mt-U1F	6-FAM-TGTAAAACGACGGCCAGTCCACCATCAGCACCCAAAGCT	39	53.8	56
mt-U2R	CAGGAAACAGCTATGACCTGGCCCTGAAGTAAGAACCAG	39	51.3	54
mt-HV2F	6-FAM-TGTAAAACGACGGCCAGTGGTCTATCACCCTATTAACCAC	40	47.5	53
mt-HV2R	CAGGAAACAGCTATGACCGCTTTGAGGAGGTAAGCTAC	38	50	53

Table 1.2: Sequences including M13 tails (underlined) and dye labels of the avian primer set

Name	Sequence (5' to 3')	Primer length (bp)	GC%	T_m (°C)
mt-Bd6F	VIC-TGTAAAACGACGGCCAGTAGGACTACGGCTTGAAAAGCC	39	51.3	54
mt-Bd7F	VIC-TGTAAAACGACGGCCAGTAGACCTACGGCTCGAAAAGCC	39	53.8	56
mt-Bd6R	CAGGAAACAGCTATGACCGATGTGCCTGACCGAGGAAC	38	55.3	56
mt-Bd7R	CAGGAAACAGCTATGACCGATGTGCCTGACCGAGGAAC	38	55.3	56

2.5. PCR optimization and capillary electrophoresis

PCR reactions were carried out using 1xPCR Gold Buffer and 1.5mM MgCl (Applied Biosystems; Roche, Branchburg; New Jersey), 0.2mM dNTP mix (Thermo Fisher Scientific Inc., Epsom: Surrey) and 2.5U Super-Therm GOLD Taq Polymerase (Southern Cross Biotechnology, Claremont; Cape Town) and 1 µl of extract (optimized at a concentration of 1 pg/µl) in 25 µl reaction volumes. All PCR reactions were carried out on the Veriti 96-Well Thermal Cycler (Applied Biosystems, Warrington; UK) under the following conditions: 94ºC for 11 minutes followed by 32 cycles of 94ºC for 1 minute, 56ºC for 1 minute, 72ºC for 1.5 minutes, and a final extension step at 72ºC for 30 minutes. The PCR was not completely successful and the larger fragments expected were not amplified. Due to this, the number of cycles was increased and it was determined that 35 cycles allowed for effective amplification. In addition the annealing temperature was decreased to 53ºC, however, this was returned to 56ºC as the lower temperature showed the amplification of unspecific peaks. After PCR, 0.5 ml of the PCR product was mixed with 10 µl HiDi®-Formamyde (Applied Biosystems) and an internal lane size standard (LIZ1200®). The samples were denatured for two minutes and were then analyzed using the 3130xl Genetic Analyzer (Applied Biosystems).

2.6. Data analysis

The data was analyzed using STRand version 2.6 (University of California). The success of the method was interpreted based on the fluorescence levels detected and the specificity of the separate peaks. The results were compared to those from Nakamura *et al.* (2009). In addition, alignments were performed between certain species and the primer sequences using BLAST (Altschul, Madden, Schanffer, Zhang, Zhang, Miller & Lipman 1997) and Geneious Software (Drummond 2010).

2.7. Sequencing

PCR products from the standard fragment analysis reactions were cleaned using the MSB spin PCRapace kit by Invitek (Invisisorb®; Berlin). Sequencing reactions were performed using the ABI PRISM® dGTP BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington; UK). Originally M13 primers were used to target the M13 tails attached to the species ID primers. Next mt-U1R and mt-U1F were used together as sequencing primers and following this mt-U1F was used alone. The amplicons were cleaned using a sodium acetate (Amresco®, Solon; Ohio) and ethanol-based method. Following this sequencing was performed on the ABI 3130xl Genetic Analyzer with 10ml Hi-Di™ Formamide (Applied Biosytems, Warrington; UK). The results were analyzed using the Applied Biosystems Sequencing Analysis 5.2.0 software.

2.8 Case Studies

2.8.1 Case Study 1

On the 5th August 2011, a sample of a meat patty was brought to the VGL for species identification. The meat patty was suspected of containing animal products of species other than bovine. The sample was extracted using a commercial kit. Universal sequencing primers mcb398 and mcb869 (Verma & Singh 2003b) were used and sequencing was performed using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington; UK) and amplicons were run on an ABI3130xl sequencer (Applied Biosystems, Warrington; UK). The results were analysed using BLASTn (Altschul *et al.* 1997). In addition the sample was tested using this species identification test. A known bovine control sample was included to confirm successful amplification in both tests.

2.8.2 Case Study 2

On the 7th of October 2010, two meat samples were brought to the VGL for species identification. These were a fillet of meat and mince meat and the laboratory was requested to determine whether the samples contained meat from an eland (*Tragelaphus oryx*). DNA was extracted using the routine tissue extraction method used in the VGL as described previously. Species identification was performed using two independent methods. Firstly universal primers mcb398 and mcb869 (Verma *et al.* 2003b) were used to sequence the samples using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington; UK) and run on a ABI3130xl Sequencer (Applied Biosystems, Warrington; UK). The results were analysed using BLASTn (Altschul *et al.* 1997). Following this, species identification based on the size variation of the mtDNA hypervariable region was performed. In both methods, a known eland sample was used as a control.

2.8.3 Case Study 3

In June 2011 two samples were submitted to the VGL. The samples were thought to be pieces of rhinoceros horn and were extracted using a commercial kit. The samples were submitted for species identification as well as genotyping using a rhinoceros-specific STR marker panel. A control was included in the test for additional verification.

CHAPTER 3: RESULTS

3.1. DNA quality and concentration

3.1.1 Sample extraction method and quality

A number of different sample-types were extracted using four different extraction methods.

Table 2: Sample types, extraction methods and DNA spectrophotometer results.

Species and Sample type	**Extraction Method**	**ng/µl**	**260/280**
Rhinoceros tissue	Commercial extraction Kit	681.21	1.81
Six hair roots	Standard Hair Extraction Method	251.61	1.36
Blood in EDTA tubes	Standard PCIA EDTA Extraction	358.27	1.90
Salted and fresh frozen tissue	Standard PCIA Tissue Extraction	1104.68	1.88

These samples were evaluated on the fragment analysis species identification method, and the electropherograms in Figures 2.1 – 2.4 show successful amplification regardless of sample or extraction type.

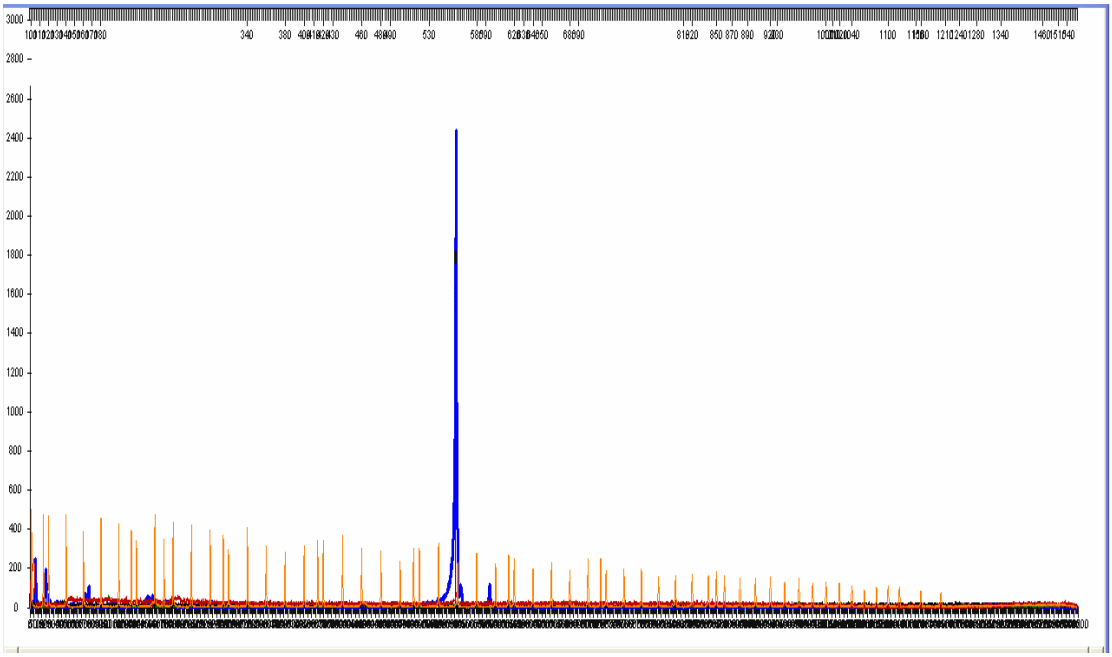

Figure 3.1: The 558bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from tissue of *Ceratotherium simum* (white rhinoceros) using a commercial extraction kit.

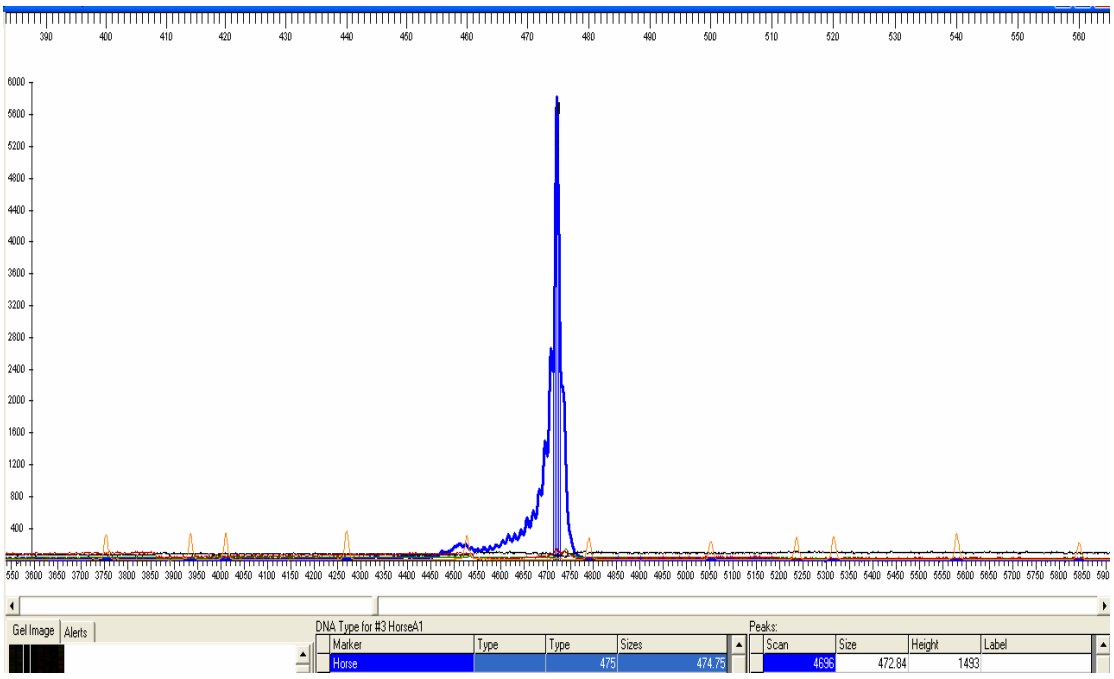

Figure 3.2: The 475bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from the hair of *Equus caballus* (horse) using the standard hair extraction method of VGL.

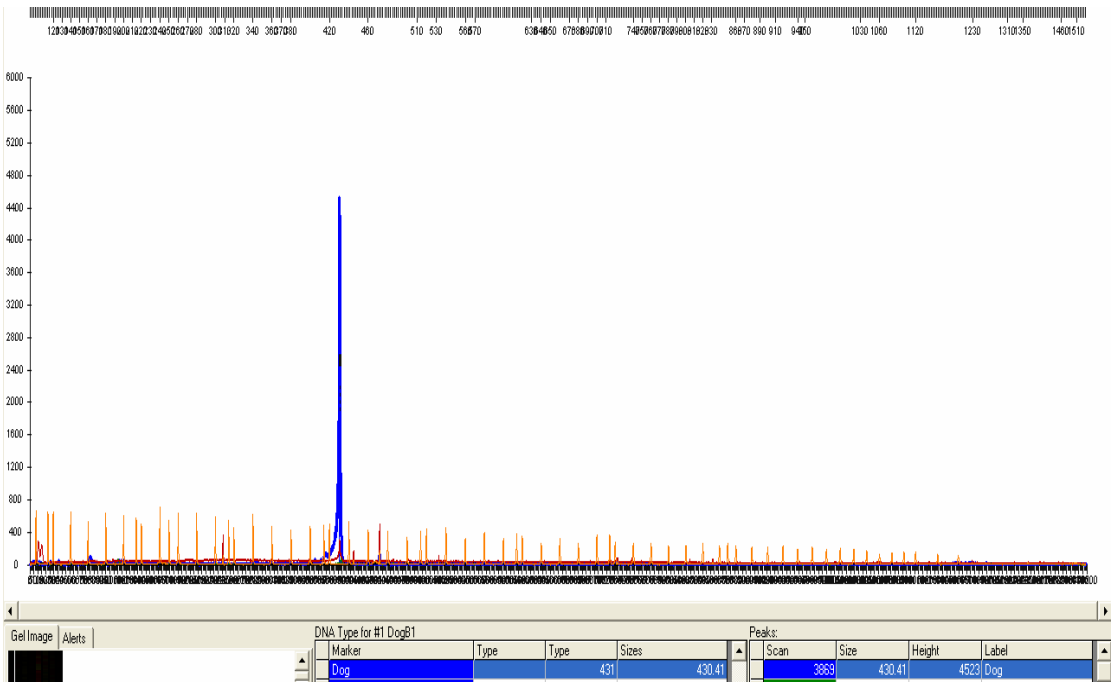

Figure 3.3: The 430bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a EDTA sample of *Canis familiaris* (domestic dog) using the standard laboratory blood extraction method of the VGL.

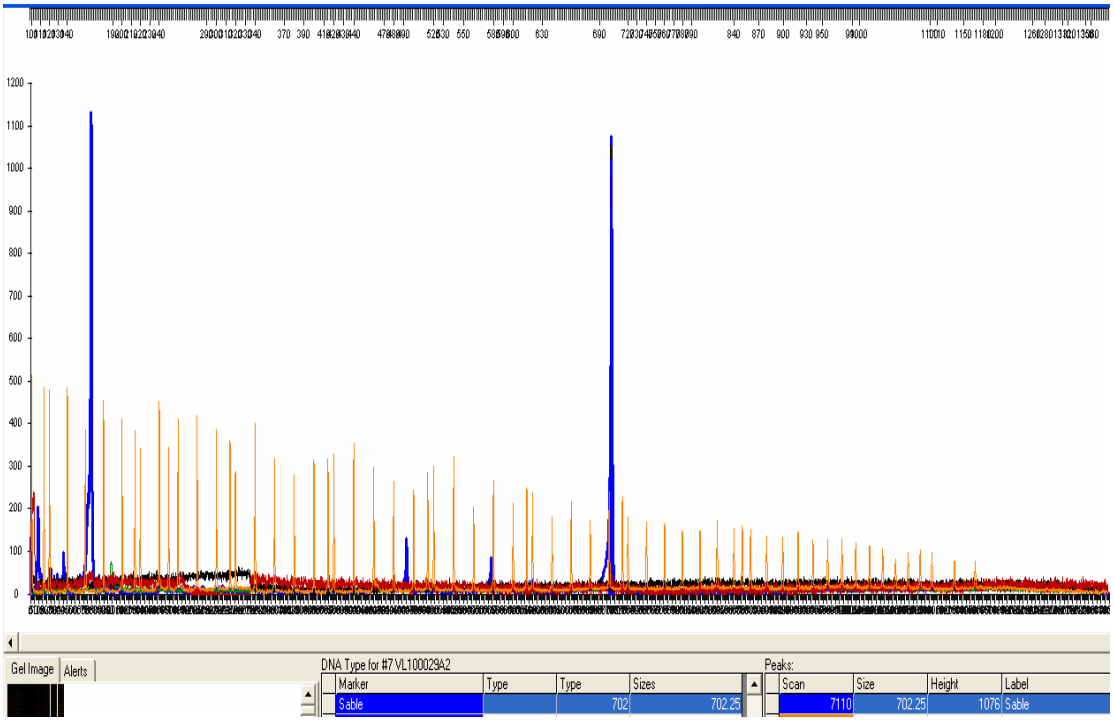

Figure 3.4: The 701bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Hippotragus niger* (sable antelope) using the standard laboratory tissue extraction method of the VGL.

A number of other sample types were examined to determine the sensitivity of this species identification system and these are summarized in Table 3 below:

Table 3: Different sample types successfully used in validating the efficiency of the species identification method.

Sample	Type
Blood	EDTA in vacutainer tubes
	Blood swab
	FTA paper slide
Tissue	Fresh tissue
	Salted tissue from taxidermist
Other	Rhinoceros horn
	Rhinoceros teeth
	Hair roots
	Buccal cells from swabs
	Elephant Ivory

3.1.2. Extract concentration optimization

Figure 2.5 shows at the DNA concentration of 10ng/µl the fluorescence was high due to a large amount of fragment amplification. This in turn caused the relative fluorescence of the size standard to be lower and made analysis of the results more difficult. The samples were diluted to 1 pg/µl and the results demonstrated the optimal dilution to be 1 pg/µl (Figure 2.6).

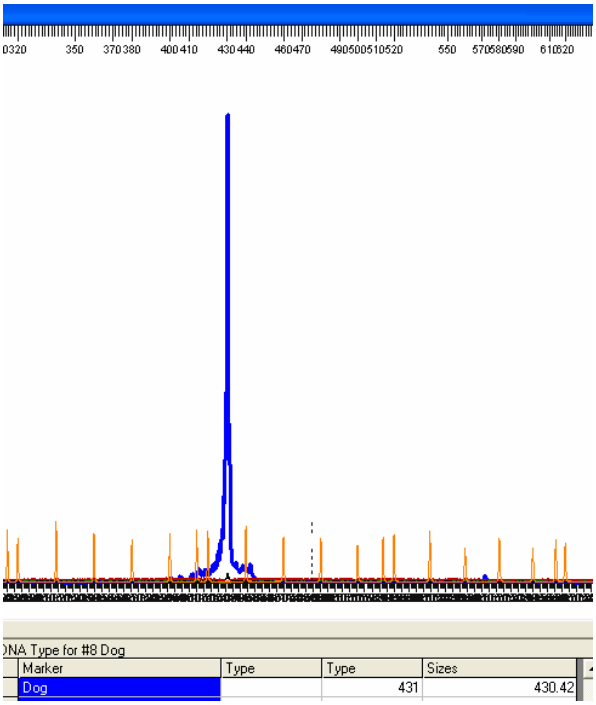

Figure 2.5: The 430bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a blood sample of *Canis familiaris*(dog) at a concentration of 10ng/µl.

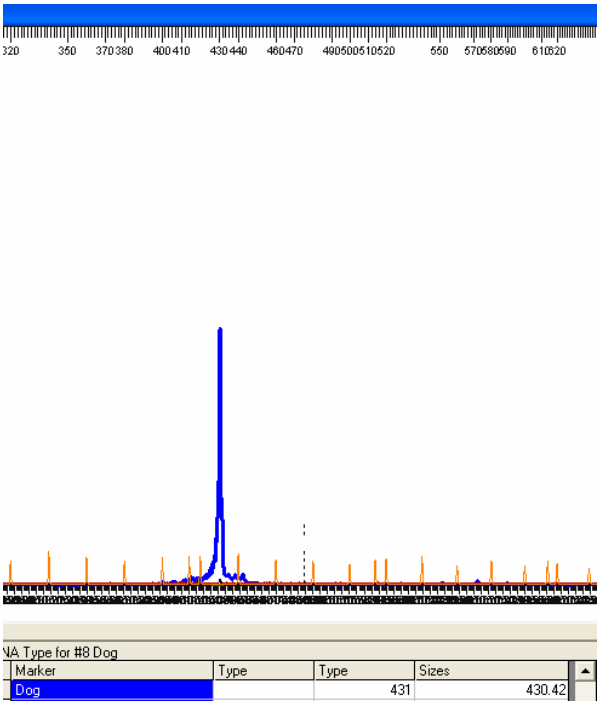

Figure 2.6: The 430bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a blood sample of *Canis familiaris*(dog) at a concentration of 1pg/µl.

3.2 Species-specific electropherograms

Thirty seven different species were each tested individually using the mammalian primer set mtU1R and mtU1F and produced electropherograms showing the fragment sizes obtained for the various species. Human samples were tested using the mammalian set as well as the human specific primer set (mt-HV2R and mtHV2F). The internal lane size standard (Liz™1200) is indicated by the orange fragments and the blue (FAM™) labelled fragment indicates the species-specific peak. The y-axis indicates the level of fluorescence and the x-axis indicates the size of the fragment (bp).

3.2.1 Domestic species

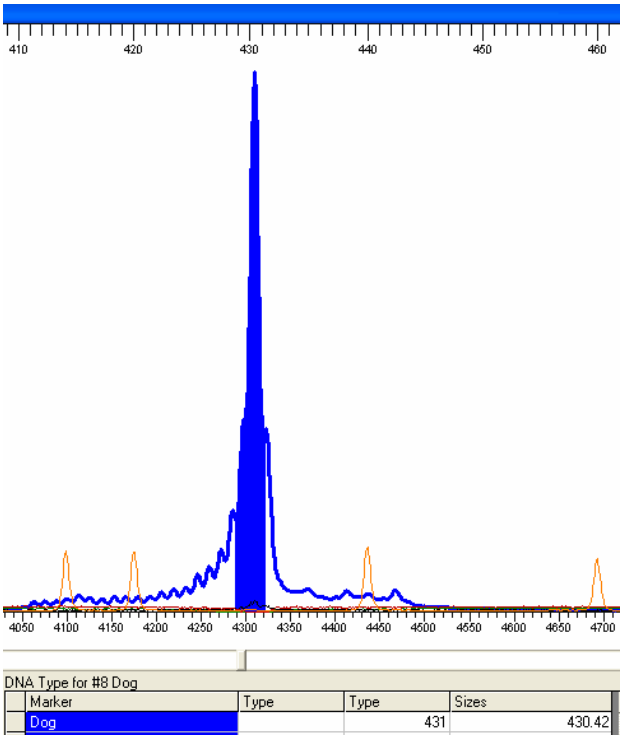

Figure 2.7: The 430bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a blood sample of *Canis familiaris* (Domestic dog).

In the case of the felid family, two fragments were constantly amplified for all species (424bp and 416bp) (Figure 2.8) in addition to other species-specific peaks. These fragments showed an increased fluorescence when compared

to the additional species-specific fragments produced by the different feline species.

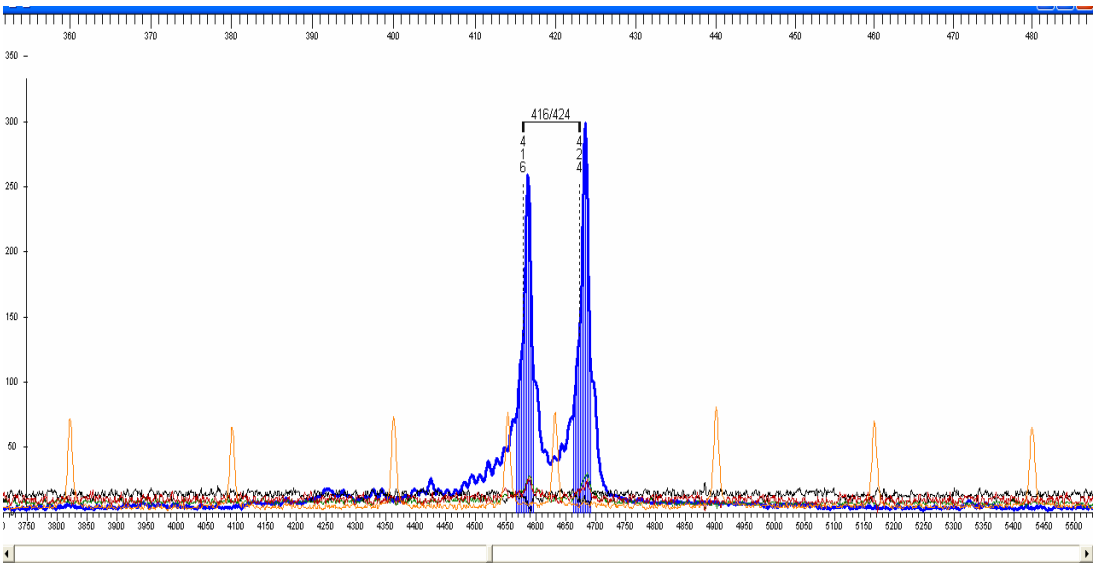

Figure 2.8: Two fragments produced using primer set mt-U1R and mt-U1F and DNA extracted from any sample of feline origin incl uding both domestic and wild species, one of 424bp and one of 416bp.

Felis catus showed amplification of two specific fragments rather than one (Figure 2.9), therefore amplified a total of 4 fragments.

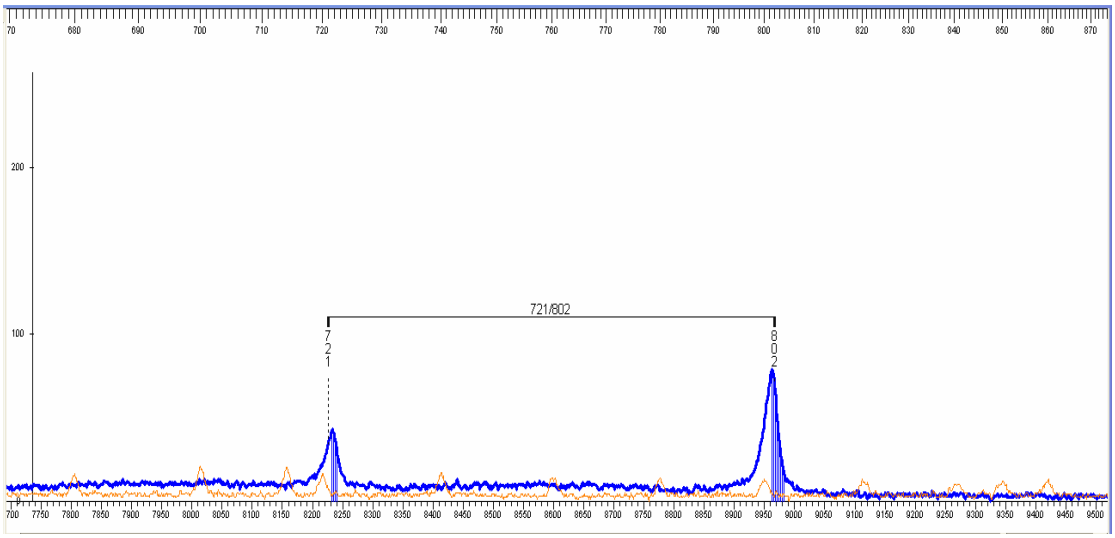

Figure 2.9: Two specific fragments produced using primer set mt-U1R and mt-U1F and DNA extracted from an EDTA sample of *Felis catus*, one of 721bp and another of 802bp.

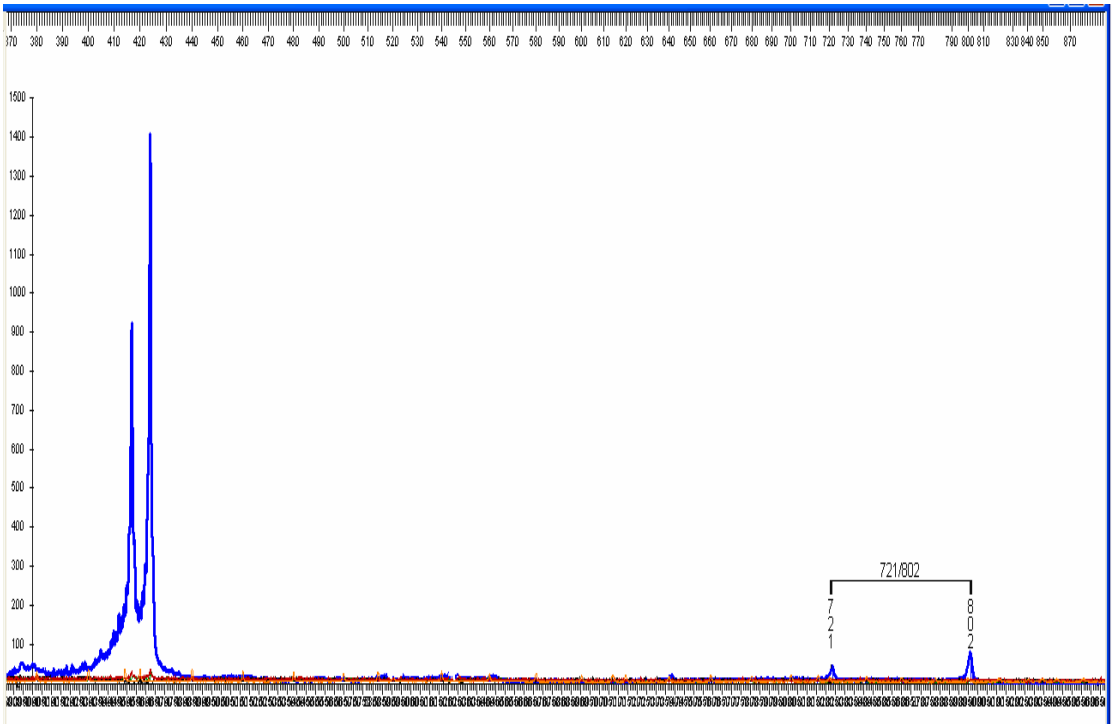

Figure 2.10: Four fragments produced using primer set mt-U1R and mt-U1F and DNA extracted from an EDTA sample of *Felis catus*, the feline specific fragments of 424bp and 416bp and the *Felis catus* (domestic cat) specific 721bp and 802bp fragments.

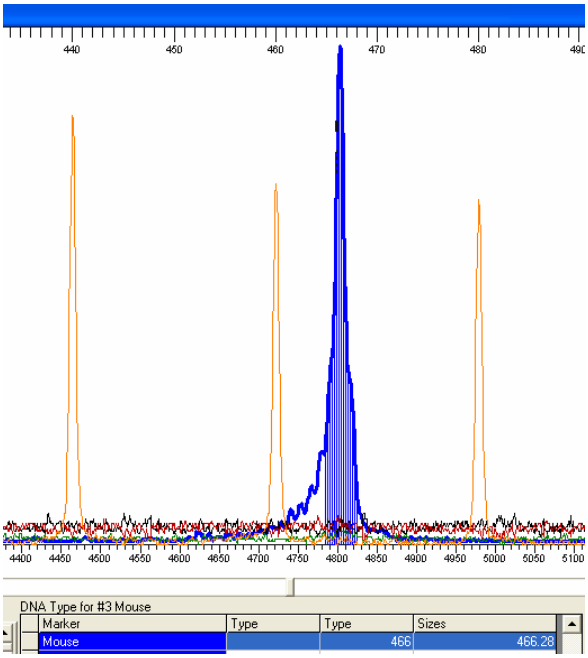

Figure 2.11: The 466bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from an EDTA sample of *Mus musculus* (mouse).

A 495bp fragment was amplified for both *Capra hircus* (domestic goat) and *Ovis aries* (domestic sheep) (Figure 2.12), however, there was an additional amplification of a specific fragment for each species, 821bp for sheep (Figure 2.15) and 846-848bp (Figure 2.13) for goat.

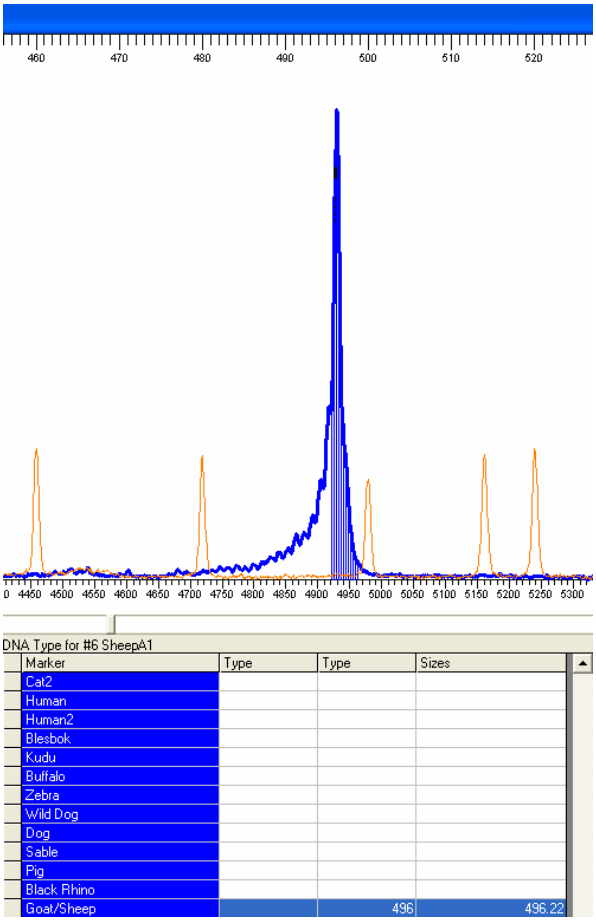

Figure 2.12: The 495bp common fragment produced when using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Capra hircus* (domestic goat) or *Ovis aries* (domestic sheep).

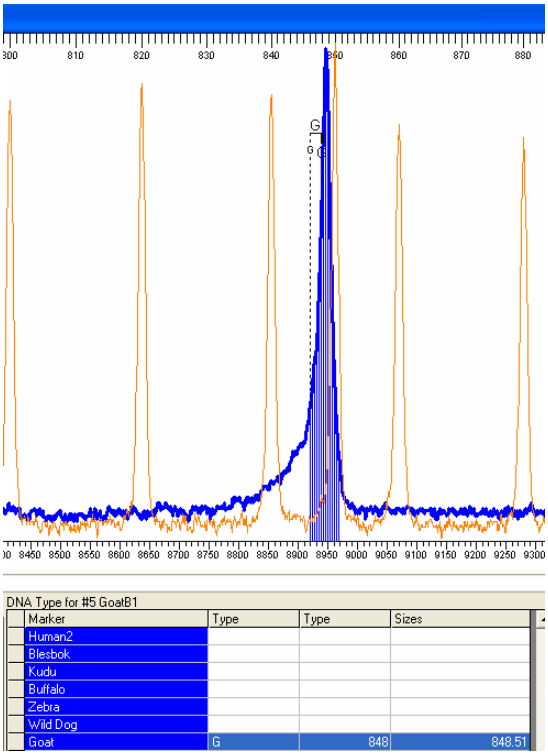

Figure 2.13: The 848bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Capra hircus* (domestic goat).

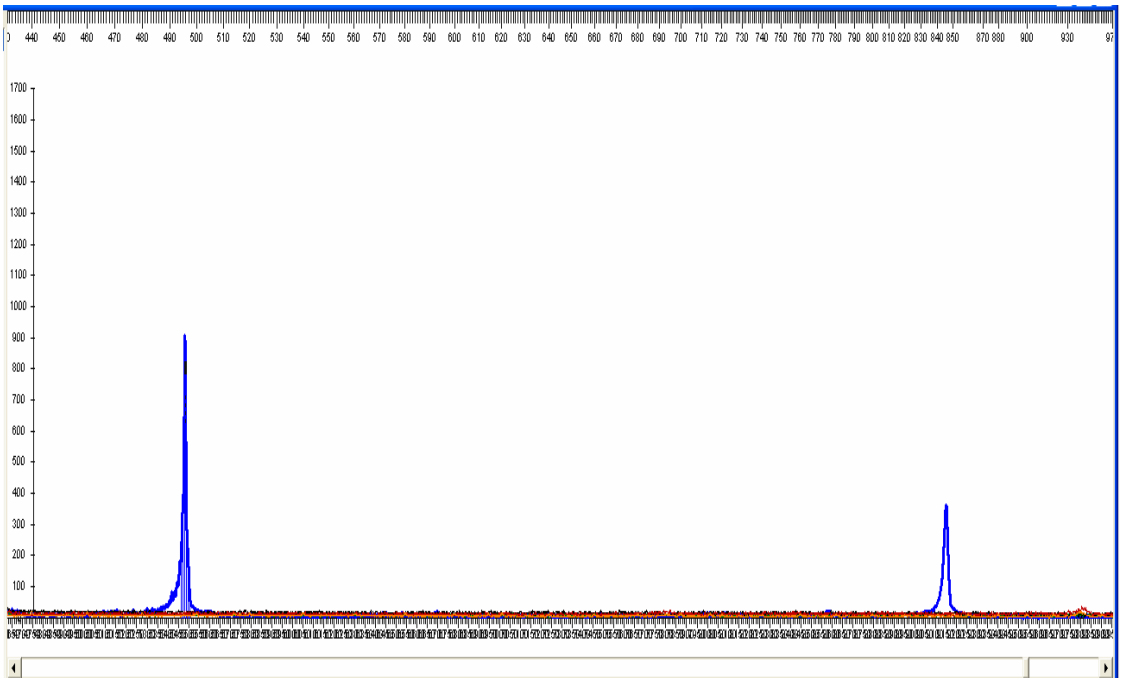

Figure 2.14: The 495bp and 848bp fragments produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Capra hircus* (domestic goat).

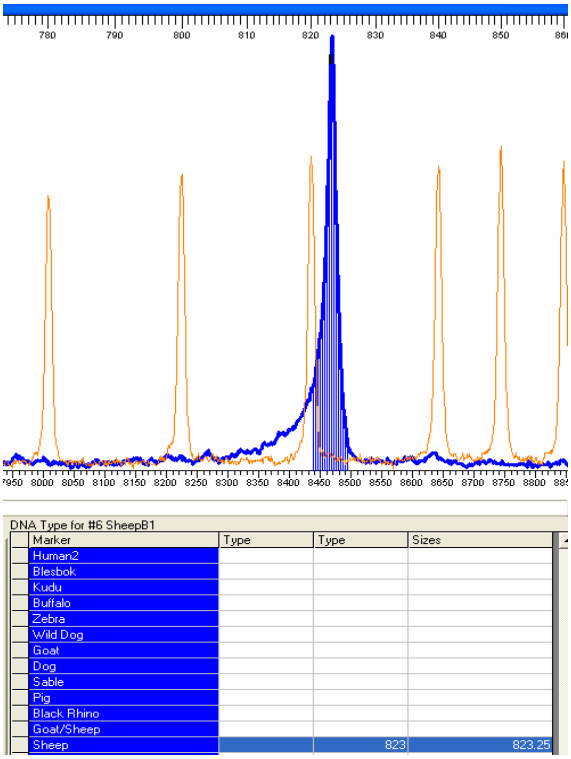

Figure 2.15: The 821bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Ovis aries* (domestic sheep).

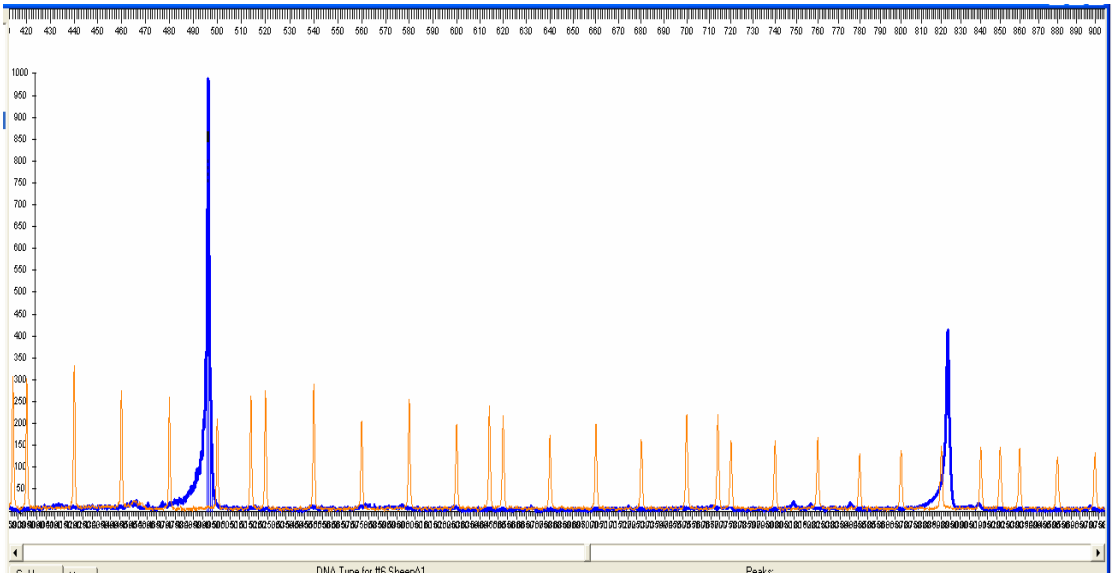

Figure 2.16: The 495bp and 821bp fragments produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Ovis aries* (domestic sheep).

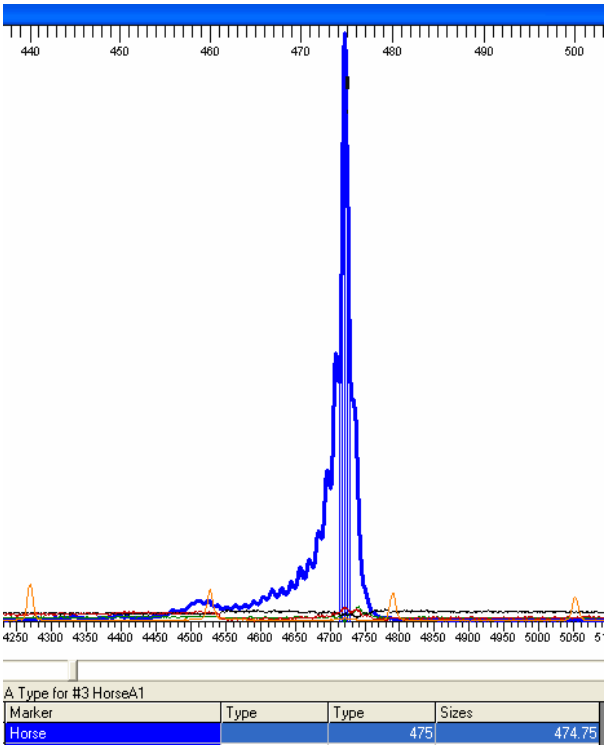

Figure 2.17: The 475bp fragment produced using primer set mt-U1R and mt-U1F DNA extracted from a tissue sample of *Equus caballus* (horse).

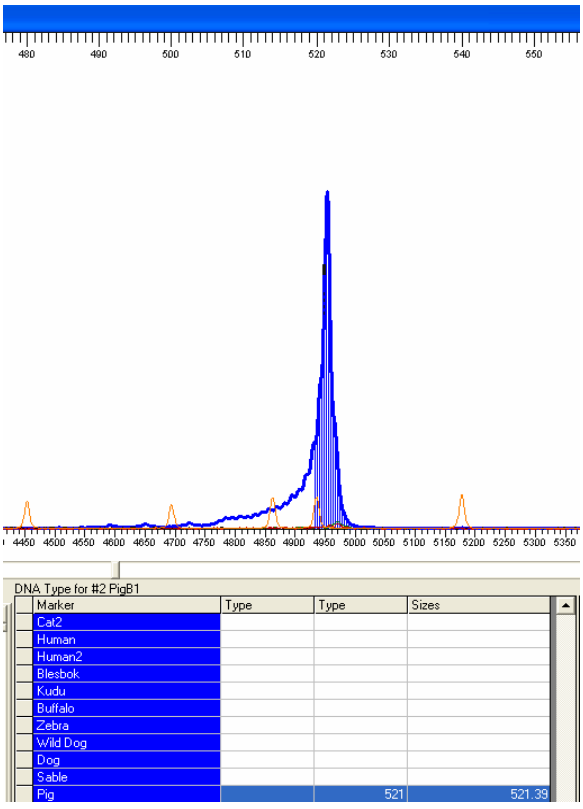

Figure 2.18: The 521bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Sus scrofa* (domestic pig).

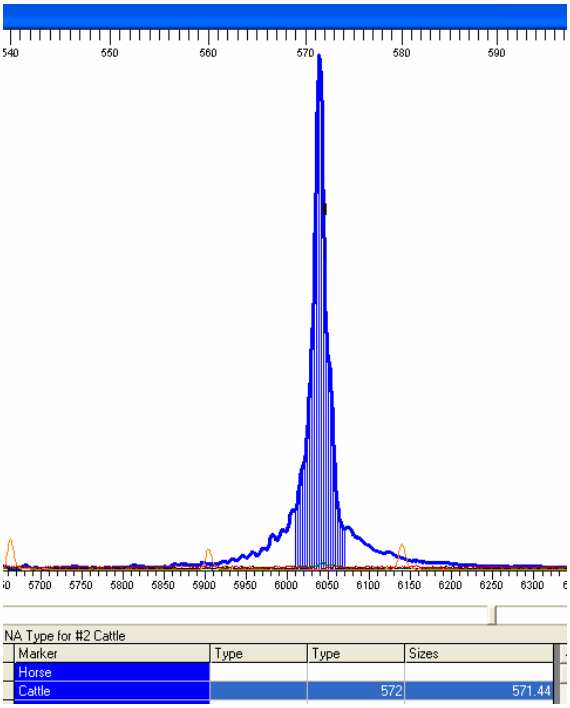

Figure 2.19: The 572bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Bos indicus* or a buccal cell sample of *Homo sapiens* (domestic cattle or human).

3.2.2 Wildlife species

3.2.2.1 Wild Antelope

A number of different wild antelope were applied to this species identification method as they could potentially form a large part of the meat trade.

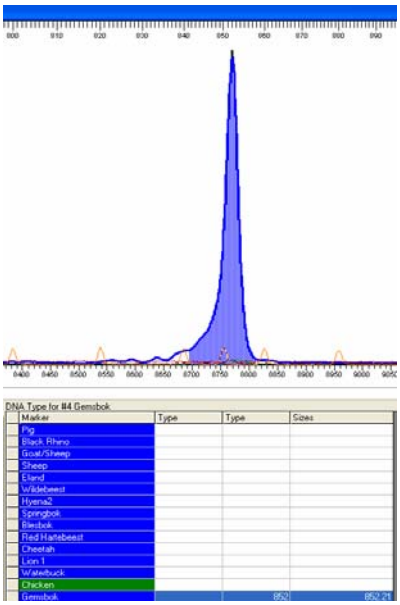

Figure 2.20: The 852bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Oryx gazelle* (gemsbok).

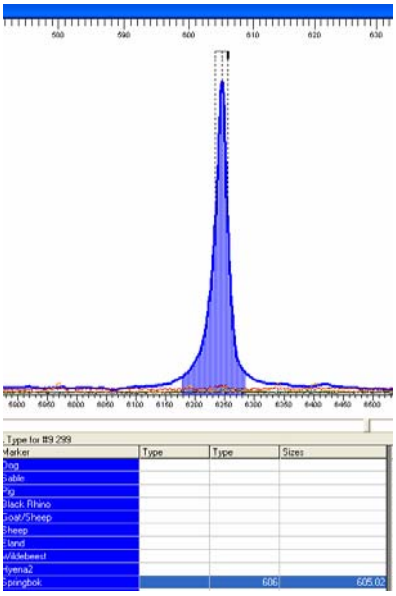

Figure 2.21: The 605bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Antidorcas marsupialis* (springbok).

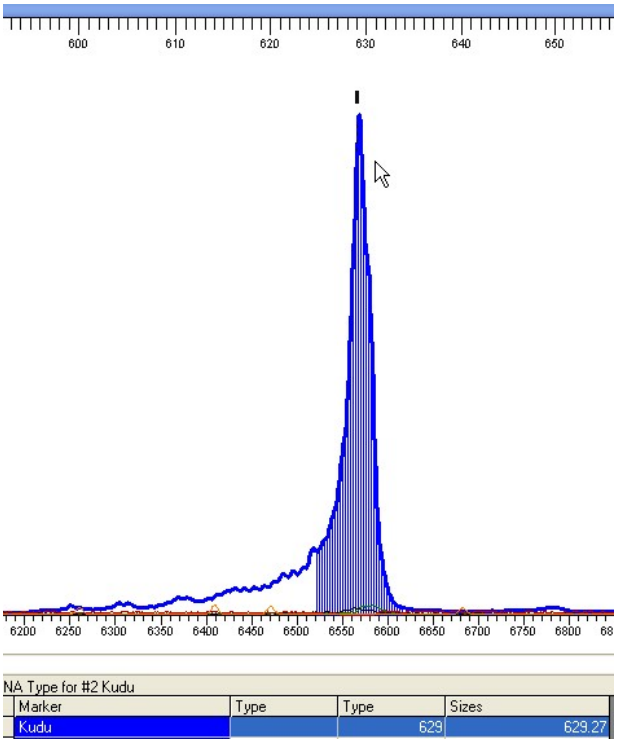

Figure 2.22: The 629bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Tragelaphus strepsiceros* (kudu).

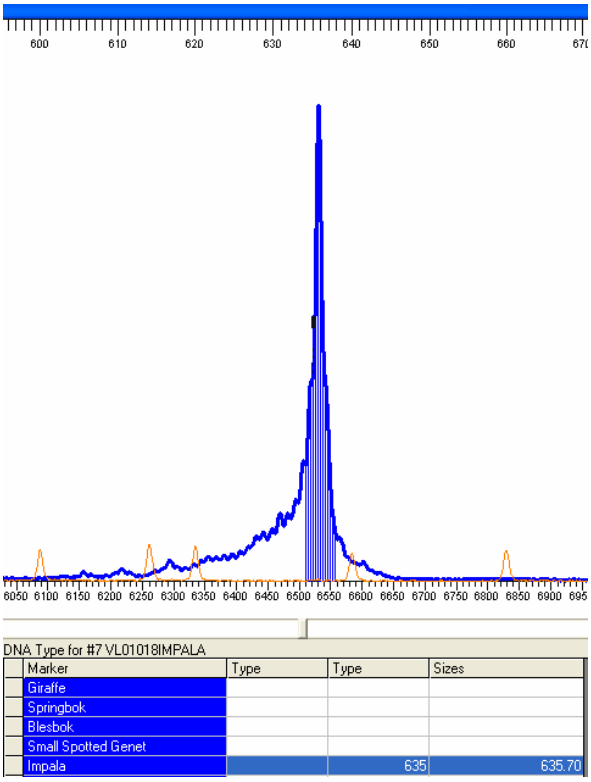

Figure 2.23: The 624bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Aepyceros melampus* (impala).

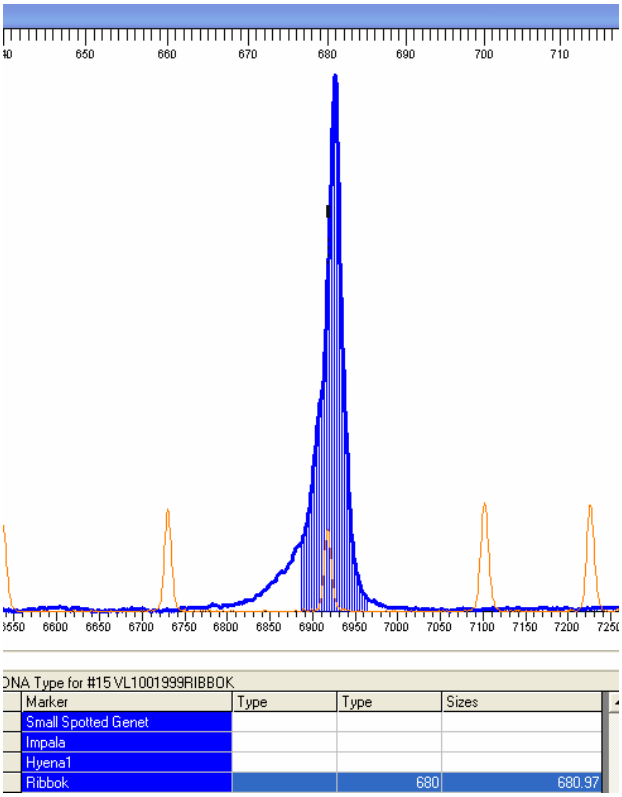

Figure 2.24: The 680bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Pelea capredus* (rhebok)

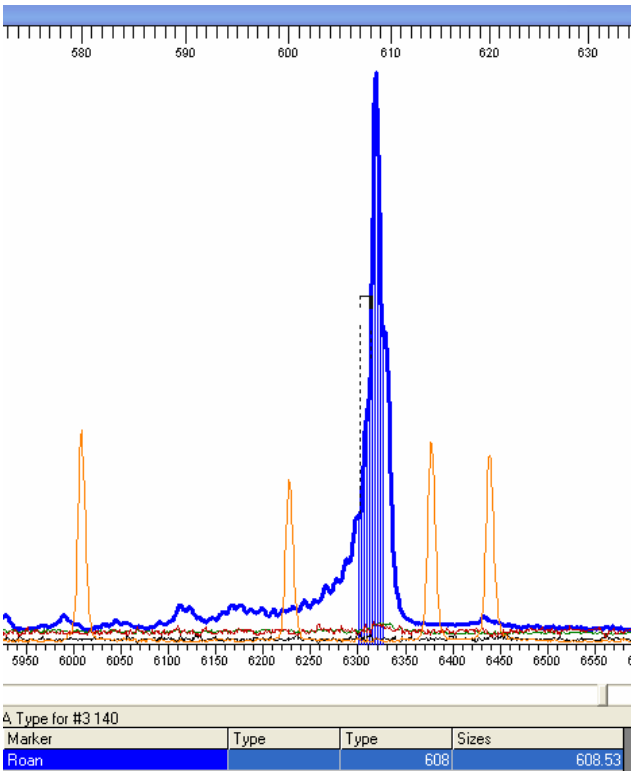

Figure 2.25: The 508bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Hippotragus equinus* (roan)

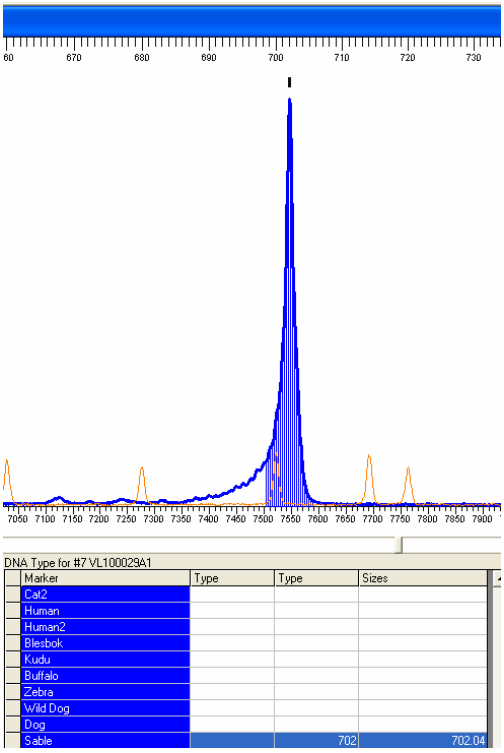

Figure 2.26: The 701bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Hippotragus niger* (sable)

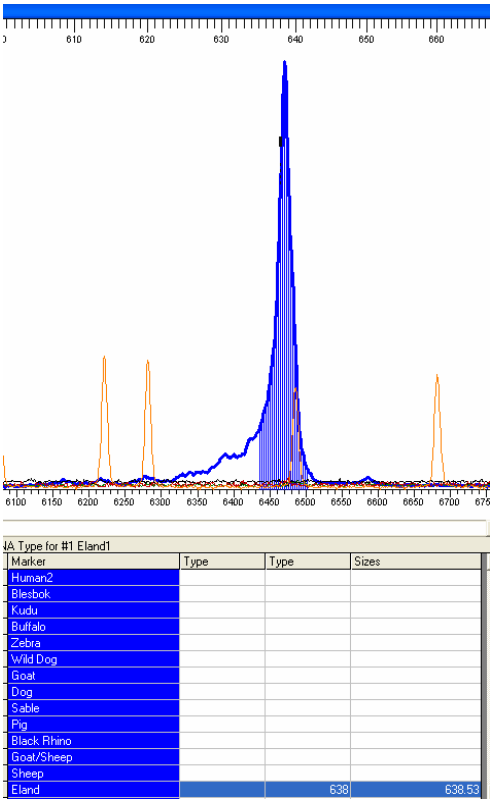

Figure 2.27: The 638bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Taurotragus oryx* (eland).

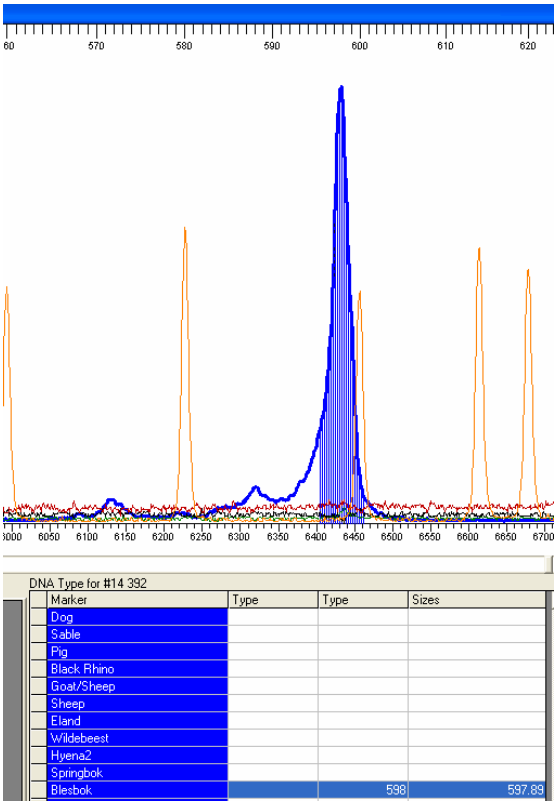

Figure 2.28: The 598bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Damaliscus phillipsi* (blesbok).

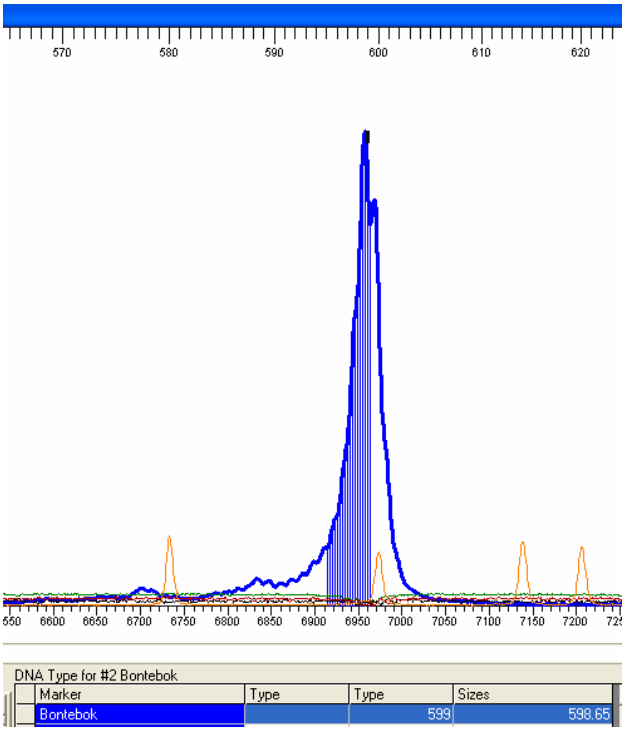

Figure 2.29: The 599bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Damaliscus pyragargus* (bontebok).

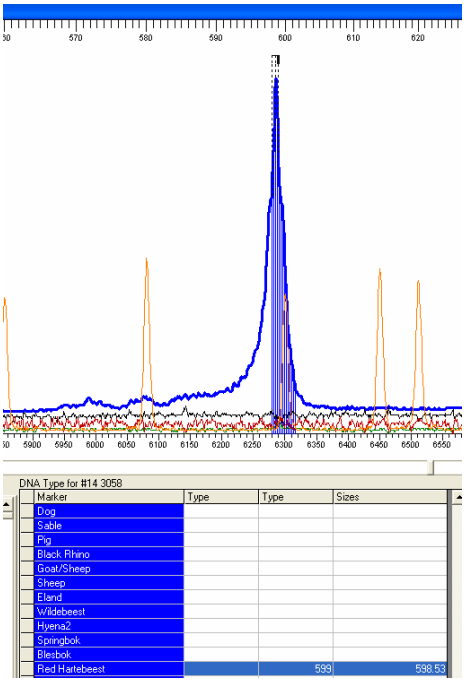

Figure 2.30: The 599bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Alcelaphus bucelaphus* (red hartebeest).

Blesbok, bontebok and red hartebeest were shown to share the fragment size of 597-599bp.

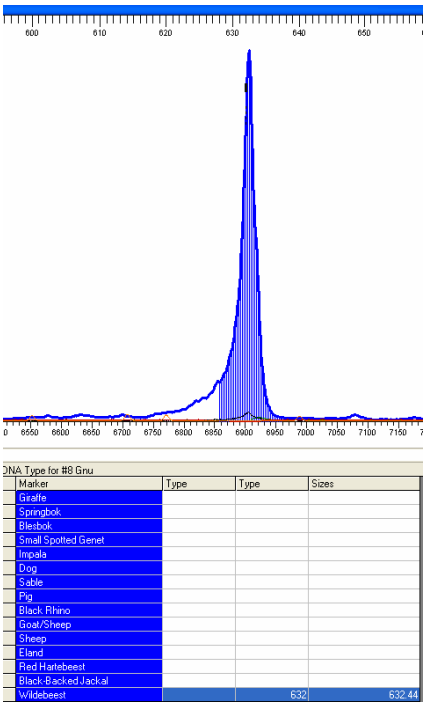

Figure 2.31: The 632bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Connochaetes taurinus* (wildebeest).

3.2.2.2 Wild feline species

In addition to the two fragments that were constantly amplified for all feline species (424bp and 416bp) (Figure 2.8), each different species showed amplification of a specific peak that allowed differentiation between them.

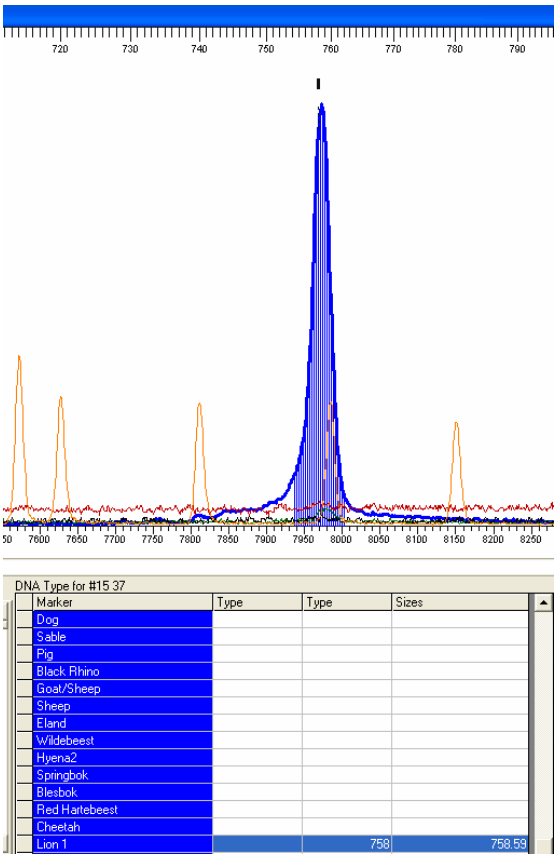

Figure 2.32: The 758bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Panthera leo* (lion).

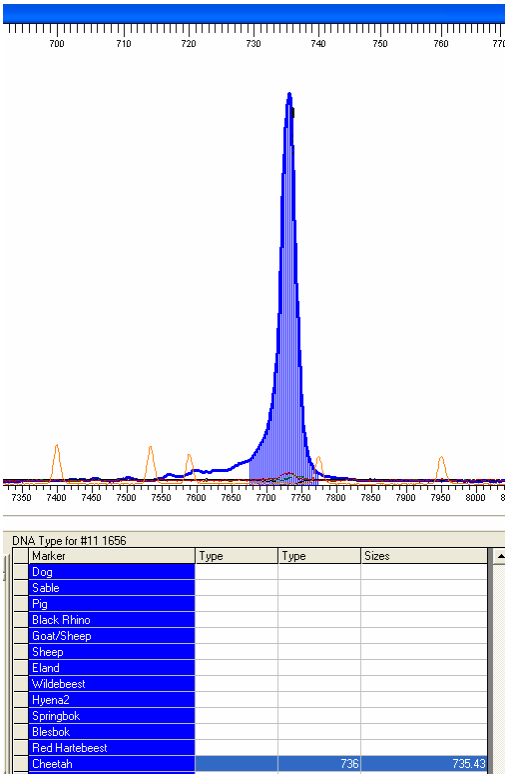

Figure 2.33: The 735bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Acinonyx jubatas* (cheetah).

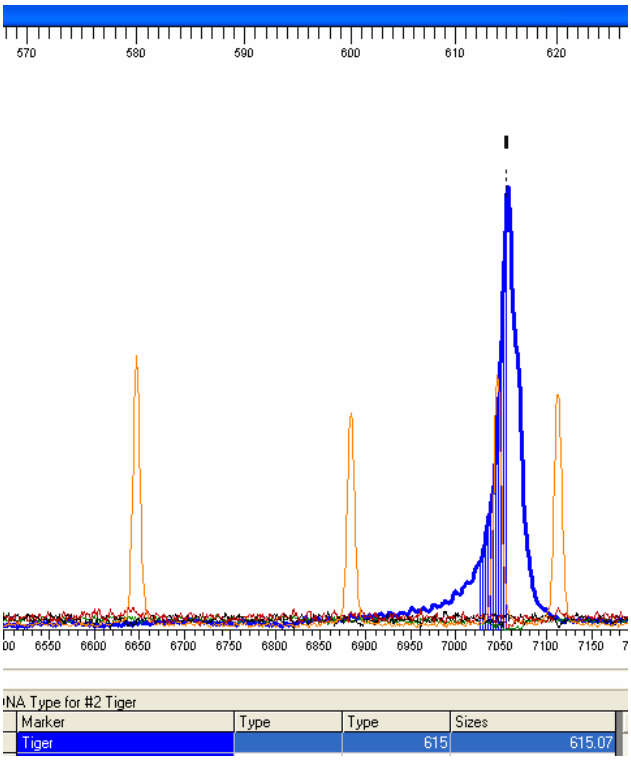

Figure 2.34: The 615bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Panthera tigris* (tiger).

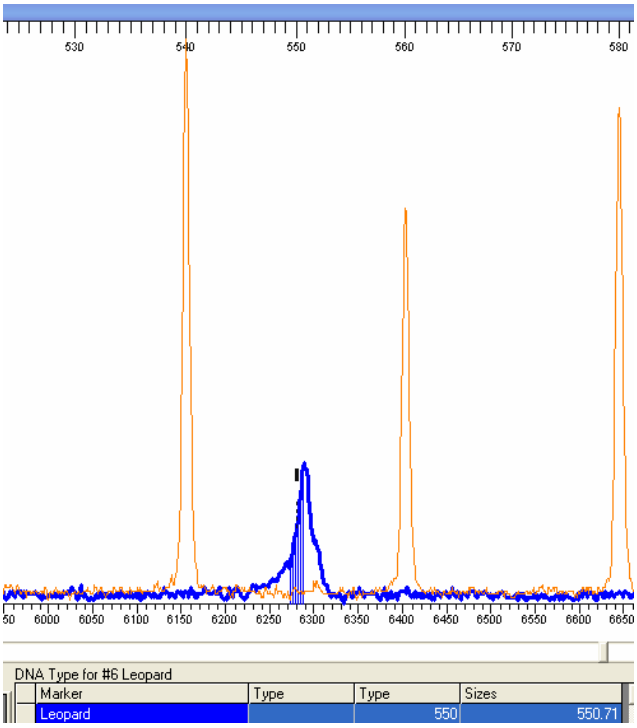

Figure 2.35: The 550bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Panthera pardus* (leopard).

3.2.2.3 Canidae and Hyaenidae

A selection of species including the black-backed jackal, hyena and wild dog produced species-specific peaks. The hyena produced two peaks as was previously seen in a number of species.

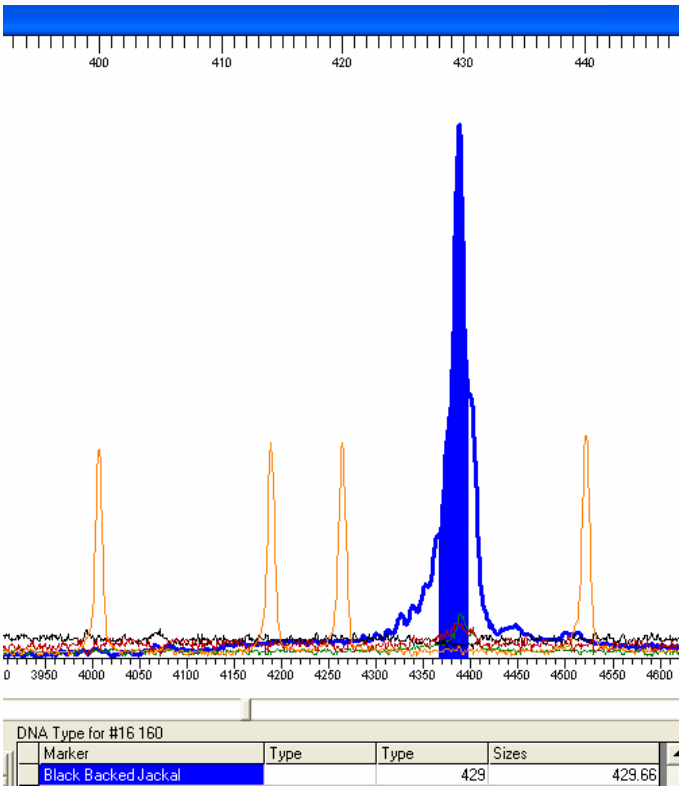

Figure 2.36: The 429bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Canis mesomelas* (black backed jackal).

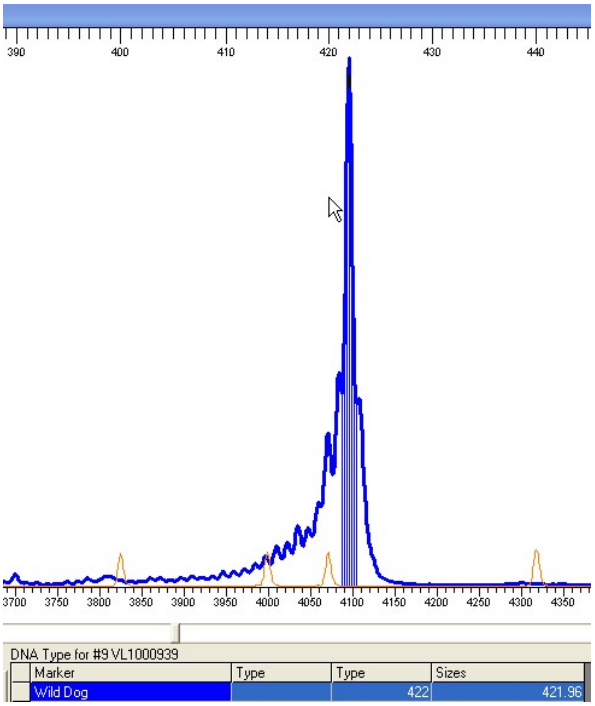

Figure 2.37: The 422bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Lycaon pictus* (wild dog).

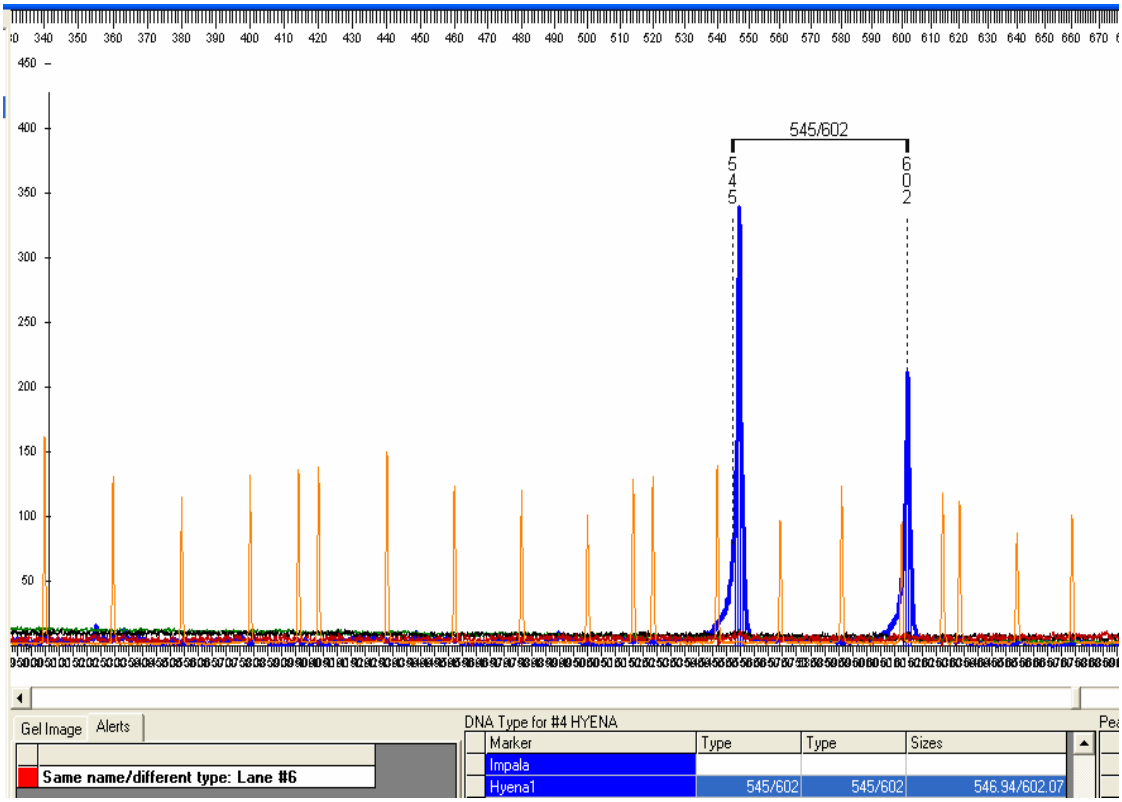

Figure 2.38: The two fragments, one of 546bp and another of 601bp, produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Crocuta crocuta* (hyena).

3.2.2.4 Other wildlife species

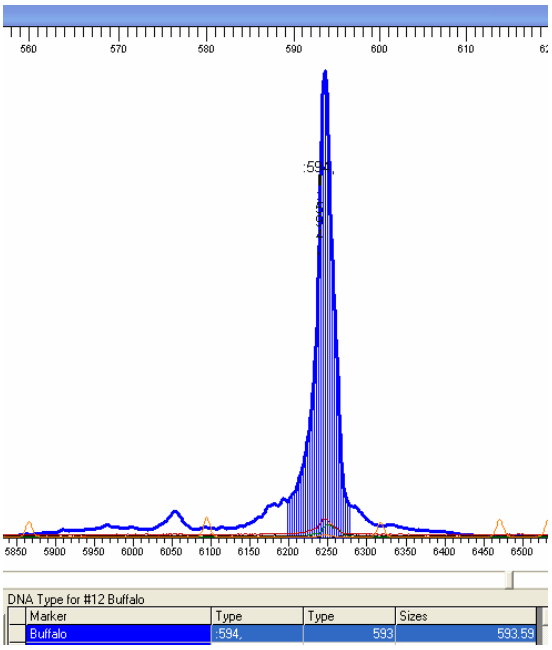

Figure 2.39: The 594bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Syncerus caffer* (buffalo).

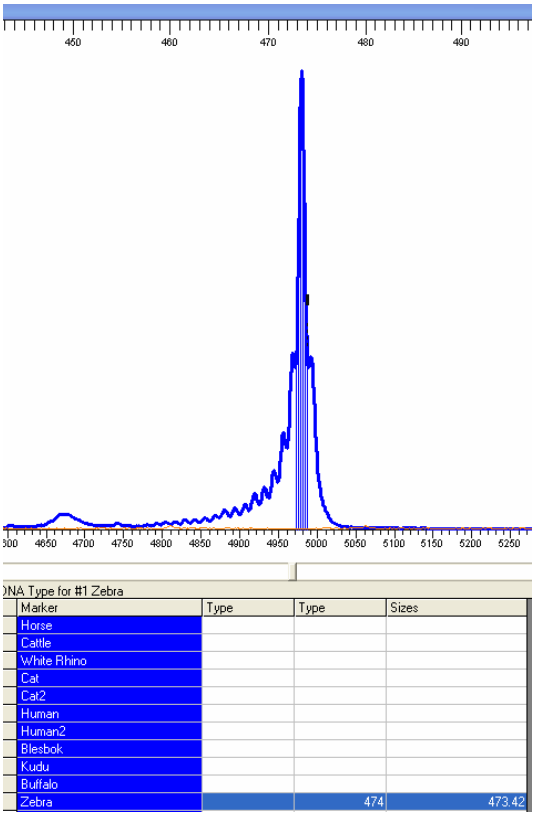

Figure 2.40: The 474bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Equus quagga* (zebra).

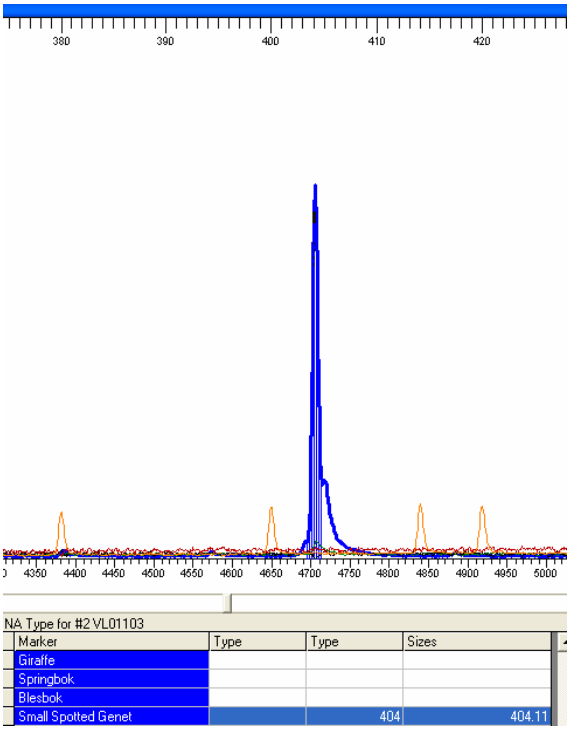

Figure 2.41: The 404bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Genetta genetta* (small spotted genet).

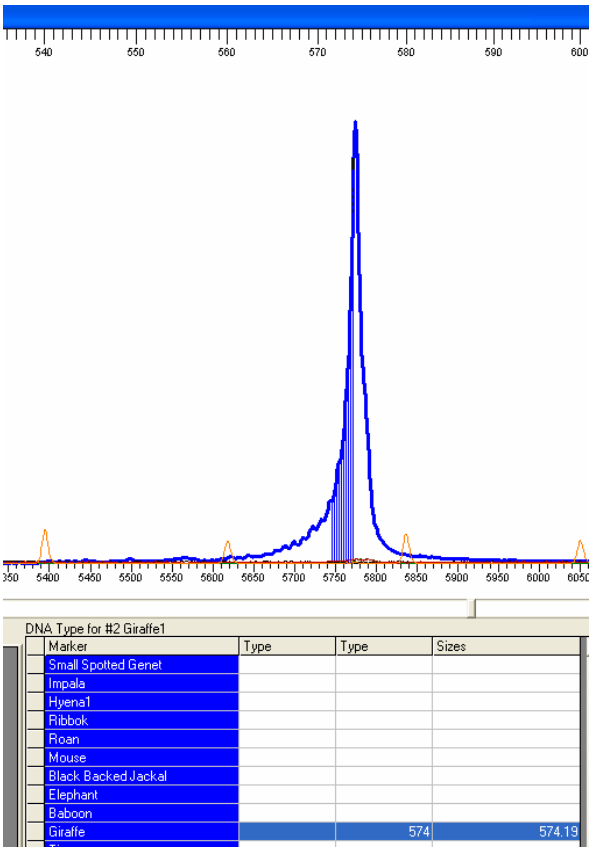

Figure 2.42: The 574bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Giraffa camelopardalis* (giraffe).

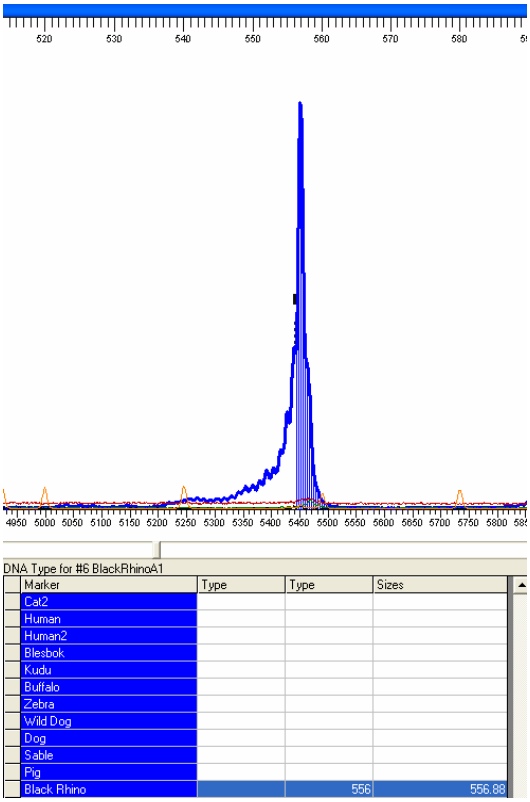

Figure 2.43: The 557bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Diceros bicornis* (black rhinoceros).

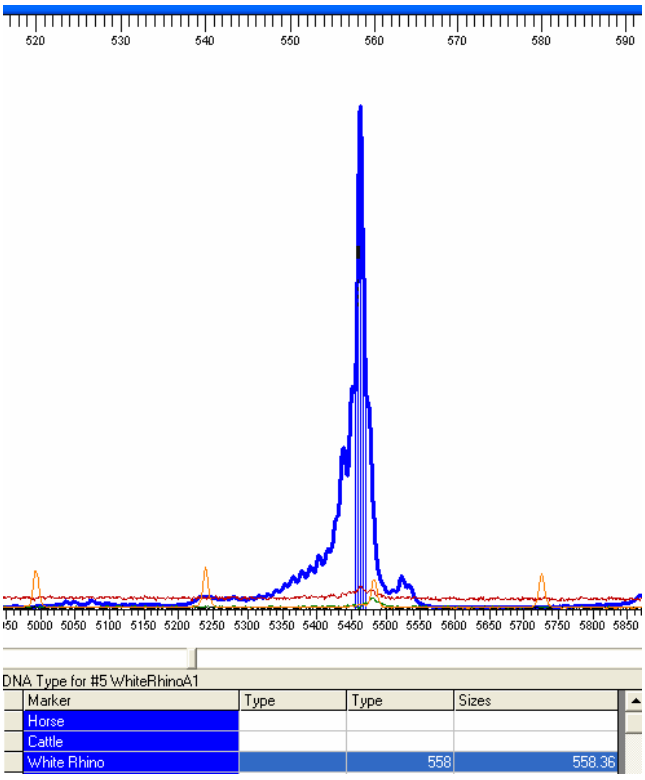

Figure 2.44: The 558bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Ceratotherium simum* (white rhinoceros).

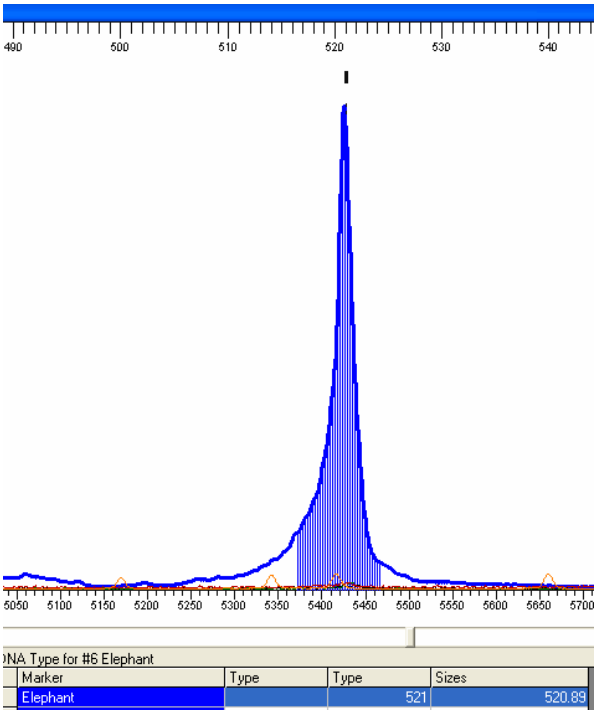

Figure 2.45: The 521bp fragment produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Loxodonta africana* (African elephant). This peak is the same size as that of the domestic pig.

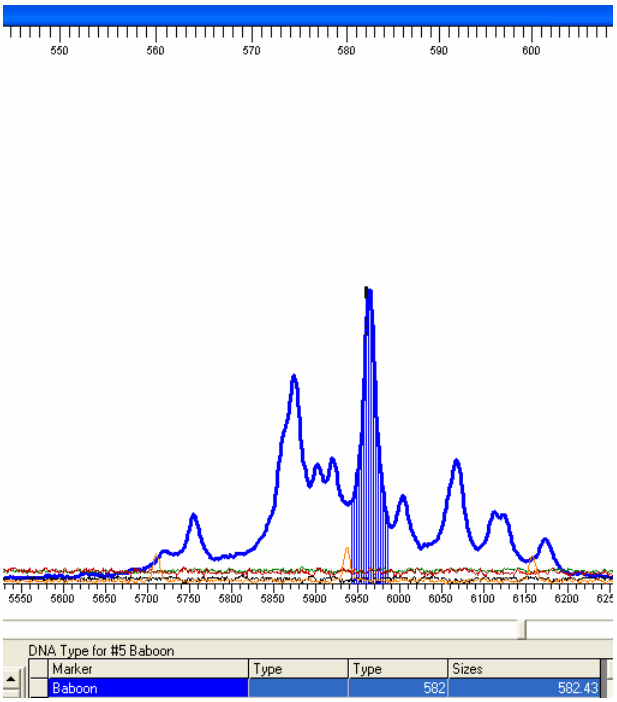

Figure 2.46: The 582bp fragment and extraneous peaks produced using primer set mt-U1R and mt-U1F and DNA extracted from a tissue sample of *Papio ursinus* (baboon). This peak was reproducible and the results did not differ despite attempting to optimize PCR conditions.

3.2.3 Human

Nakamura *et al.* 2010 described the use of two primer pairs in order to distinguish between human and cattle which amplify the same size fragment using only primer pair mt-U1R and mt-U1F. The second primer pair mt-HV2R and mt-HV2F were used to amplify the human specific peaks 624bp and 576bp (Figure 2.47).

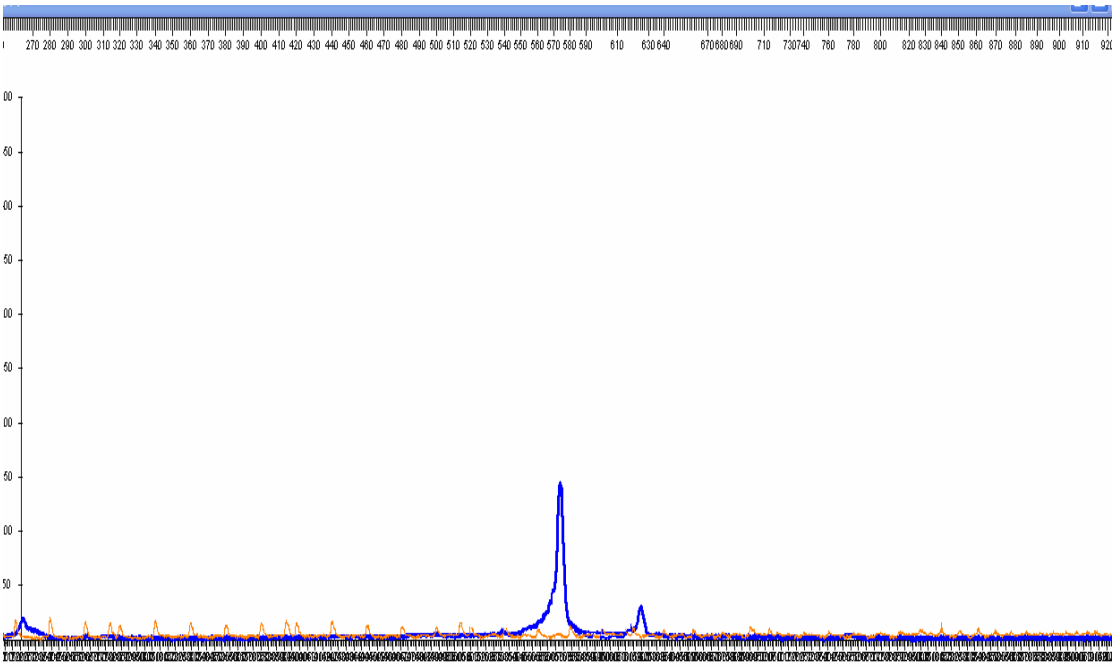

Figure 2.47: The two fragments produced using primer set mt-U1R and mt-U1F, primer set mt-HV2R and mt-HV2F and *Homo sapien* DNA, one of 576bp and one of 624bp

3.2.4 Avian species

The primer pairs for the bird species were tested on chicken and a species-specific fragment of size 622bp was obtained (Figure 2.48). In order to ensure that the primers were specific to avian species, a number of mammalian species including canine, bovine, ovine and feline were used as negative controls. No amplification of these samples was noted (Figure 2.49).

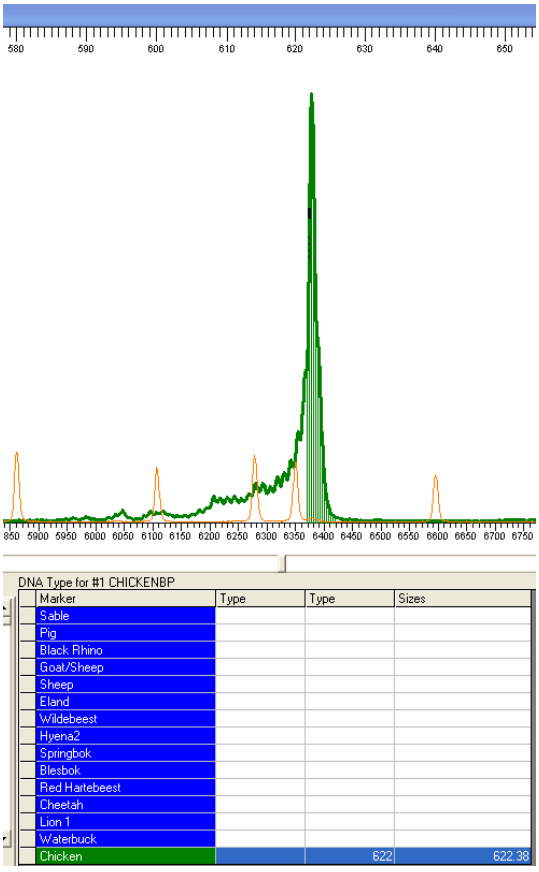

Figure 2.48: The 622bp fragment produced using primer sets mt-Bd6F, mt-Bd6R, mt-Bd7F and mt-Bd7R and DNA extracted from a tissue sample of *Gallus domesticus* (domestic chicken). The fragment is green as the avian primers are labelled with VIC® fluorescent dye.

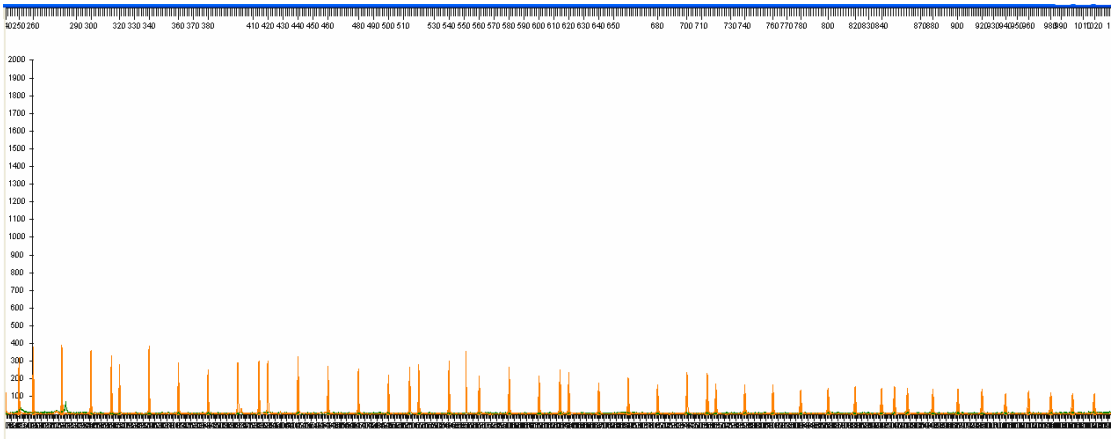

Figure 2.49: No fragments produced using primer sets mt-Bd6F and mt-Bd6R, mt-Bd7F and mt-Bd7R and a mixture of DNA extracted from a tissue samples of canine, bovine, ovine and feline (dog, cattle, pig and cat).

A total of 37 species were used in assessing the speciation method and the results are summarized in the table below.

Table 4: Summary of all the species that were identified with their corresponding fragment sizes, scientific names and common names.

	Scientific Name	Common Name	Size of fragment (bp)
1	Canis familiaris	Dog	430
2	Felis catus	Cat	721, 802, 424 and 416
3	Equus caballus	Horse	475
4	Ovis aries	Sheep	821 and 495
5	Capra hircus	Goat	846-848 and 495
6	Bos *Indicus*	Cattle	572
7	Mus musculus	Mouse	466
8	Oryx gazelle	Gemsbok	852
9	Antidorcas marsupialis	Springbok	605
10	Tragelaphus strepsiceros	Kudu	629
11	Aeryoeros melampus	Impala	624
12	Pelea caprelus	Rhebok	680
13	Hippotragus equines	Roan	508
14	Hippotragus niger	Sable	701
15	Tragelaphus oryx	Eland	638
16	Connochaetes taurinus	Wildebeest	632-633
17	Syncerus caffer	Buffalo	594
18	Equus quagga	Zebra	474
19	Crocuta crocuta	Hyena	546 and 601
20	Genetta genetta	Small Spotted Genet	404
21	Canis mesomelas	Black Backed Jackal	429
22	Panthera leo	Lion	758, 424 and 416
23	Acinonyx jubatas	Cheetah	735, 424 and 416
24	Diceros bicornis	Black Rhinoceros	557
25	Ceratotherium simum	White Rhinoceros	558
26	Giraffa camelopardalis	Giraffe	574
27	Homo sapiens	Human	576 and 624
28	Papio ursinus	Baboon	582
29	Lycaon pictus	Wild Dog	422
30	Gallus domesticus	Chicken	622
31	Panthera pardus	Leopard	550, 424 and 416
32	Pathera tigris	Tiger	615, 424 and 416
33	Damaliscus phillipsi	Blesbok	597-599
34	Alcelaphus bucelaphus	Red Hartebeest	597-599
35	Damaliscus pyragargus	Bontebok	597-599
36	Sus scrofa	Pig	521
37	Loxodonta Africana	Elephant	521

3.3 Mixed samples

A number of sample mixtures were tested. In the first two mixtures, the concentration of DNA for all species was 1 pg/µl (Table 5). The concentrations of different species were than randomly changed (mixtures 3 – 5). The concentration of the DNA in the feline mixture was kept at 100 pg/µl (mixture 6).

Table 5: Species included in the sample mixtures and the concentrations at which they were used.

	Species Included	Concentration
Mixture 1 (figure 2.50)	Dog	1 pg/µl
	Cat	1 pg/µl
	Horse	1 pg/µl
	Goat	1 pg/µl
	Cattle	1 pg/µl
Mixture 2 (figure 2.51)	Black-backed Jackal	1 pg/µl
	Impala	1 pg/µl
	Blesbok	1 pg/µl
	Rhebok	1 pg/µl
	Sable	1 pg/µl
Mixture 3 (figure 2.52)	Blesbok	1 pg/µl
	Goat	1 pg/µl
	White Rhinoceros	100 pg/µl
	Bontebok	1 pg/µl
	Red Hartebeest	1 pg/µl
	Cat	50 pg/µl
	Horse	1 pg/µl
	Dog	50 pg/µl
Mixture 4 (figure 2.53)	Black-backed Jackal	1 pg/µl
	Cat	100 pg/µl
	Cheetah	100 pg/µl
	Goat	1 pg/µl
	White Rhinoceros	100 pg/µl

	Bontebok	1 pg/µl
	Red Hartebeest	1 pg/µl
	Blesbok	1 pg/µl
Mixture 5 (figure 2.54)	Cat	100 pg/µl
	Blesbok	1 pg/µl
	Bontebok	1 pg/µl
	Red Hartebeest	1 pg/µl
	Cheetah	100 pg/µl
	Buffalo	1 pg/µl
	Black-backed Jackal	1 pg/µl
	Impala	50 pg/µl
	White Rhinoceros	50 pg/µl
Mixture 6 (figure 2.55)	Cat	100 pg/µl
	Lion	100 pg/µl
	Cheetah	100 pg/µl
	Tiger	100 pg/µl
	Leopard	100 pg/µl

The first mixture tested was a mixture of common domestic species (Figure 2.50). The DNA concentration for all the species included in the mixture was 1pg/µl. Following this, a number of wildlife species were tested, also at a DNA concentration of 1pg/µl (Figure 2.51).

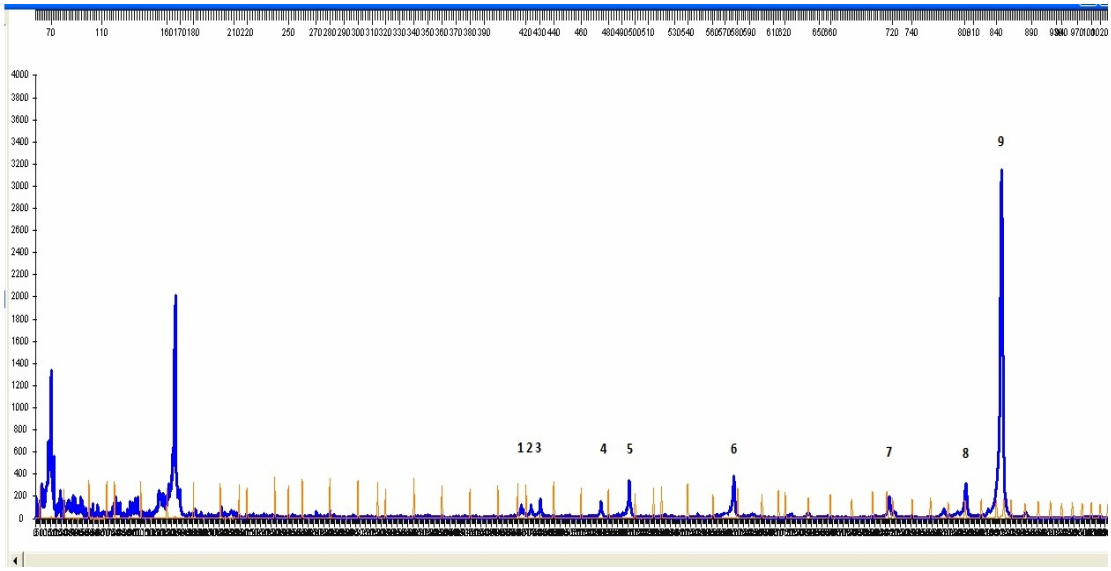

Figure 2.50: Mixture 1. The fragments produced using primer set mt-U1R and mt-U1F and a mixture of DNA from 5 different common domestic species. These included *Canis familiaris, Felis catus, Equus caballus, Capra hircus* and *Bos indicus.* The fragments are numerically labelled and described below:

1 – 416bp fragment produced from *Felis catus*
2 – 424bp fragment produced from *Felis catus*
3 – 430bp fragment produced from *Canis familiaris*
4 – 475bp fragment produced from *Equus caballus*
5 – 495bp fragment produced from *Capra hircus*
6 – 572bp fragment produced from *Bos indicus*
7 – 721bp fragment produced from *Felis catus*
8 – 802bp fragment produced from *Felis catus*
9 – 846bp fragment produced from *Capra hircus*

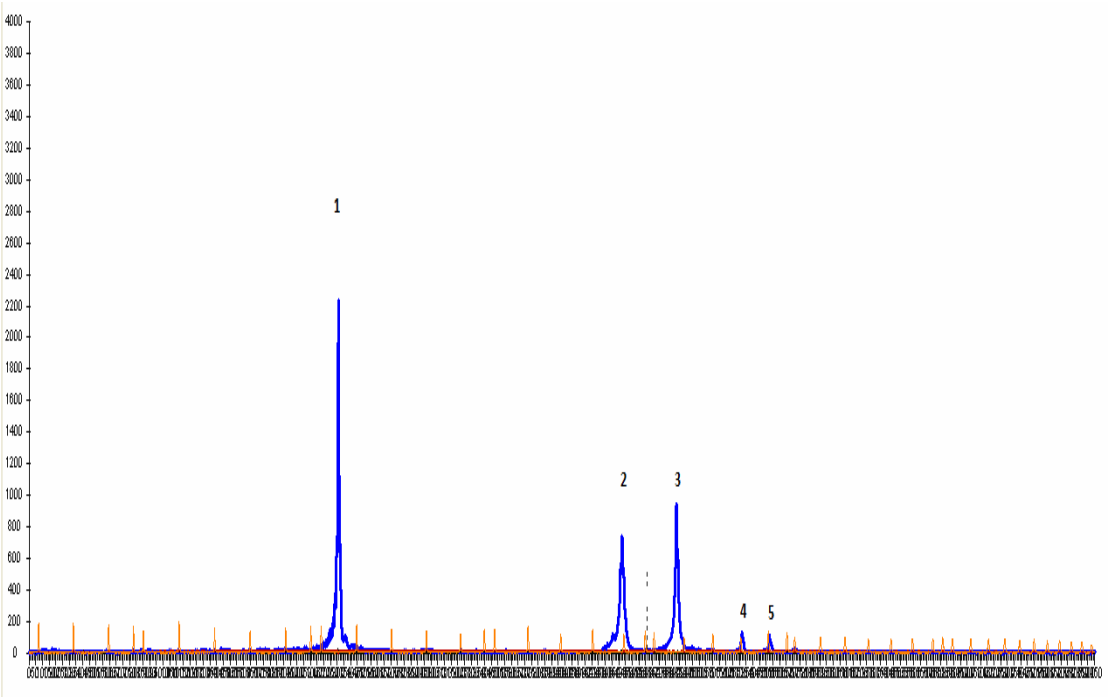

Figure 2.51: Mixture 2. The fragments produced using primer set mt-U1R and mt-U1F and a mix of 5 different wildlife species. These included *Canis mesomelas, Aeryoeros melampus, Damaliscus phillipsi, Pelea caprelus* and *Hippotragus niger.*

1 – 429bp fragment produced from *Canis mesomelas*

2 – 598bp fragment produced from *Damaliscus phillipsi*

3 – 624bp fragment produced from *Aeryoeros melampus*

4 – 680bp fragment produced from *Pelea caprelus*

5 – 701bp fragment produced from *Hippotragus niger*

In the following three mixtures the DNA concentrations between different species were changed as is detailed in Table 5.

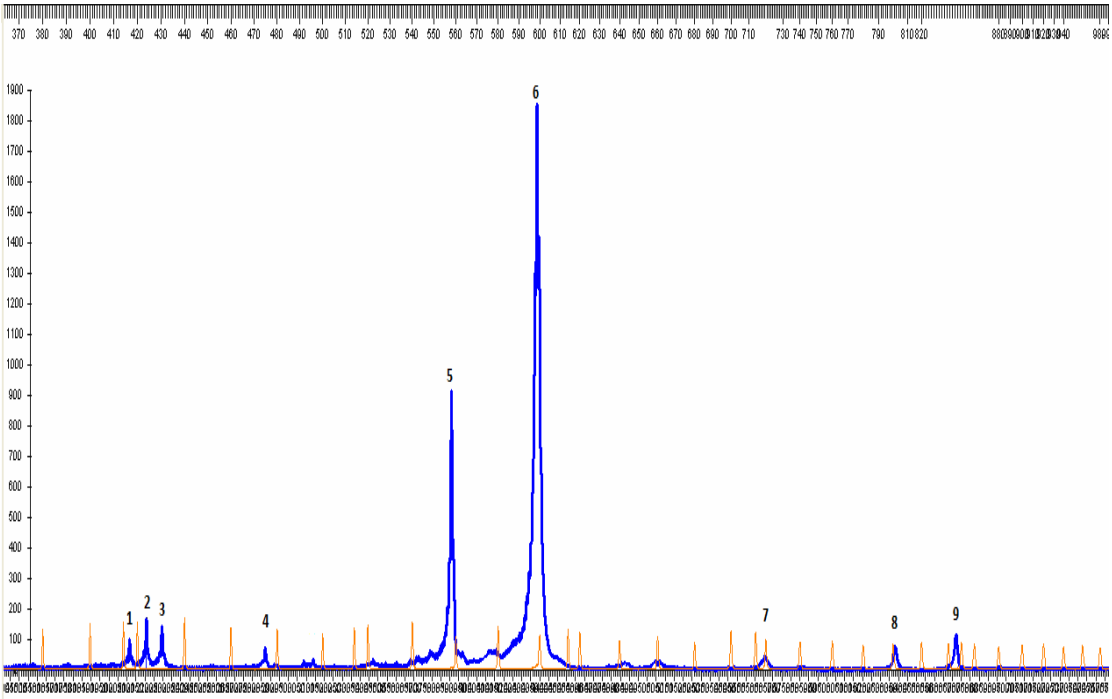

Figure 2.52: Mixture 3. The fragments produced using primer set mt-U1R and mt-U1F and a mix of 8 different species DNA at 1pg/µl. These included *Canis familiaris, Felis catus, Equus caballus, Capra hircus, Ceratotherium simum, Damaliscus pyragargus, Damaliscus phillipsi and Alcelaphus bucelaphus.*

1 – 416bp fragment produced from *Felis catus*

2 – 424bp fragment produced from *Felis catus*

3 – 430bp fragment produced from *Canis familiaris*

4 – 475bp fragment produced from *Equus caballus*

5 – 558bp fragment produced from *Ceratotherium simum*

6 – 599bp fragment produced from *Damaliscus pyragargus, Damaliscus phillipsi and Alcelaphus bucelaphus*

7 – 721bp fragment produced from *Felis catus*

8 – 802bp fragment produced from *Felis catus*

9 – 846bp fragment produced from *Capra hircus*

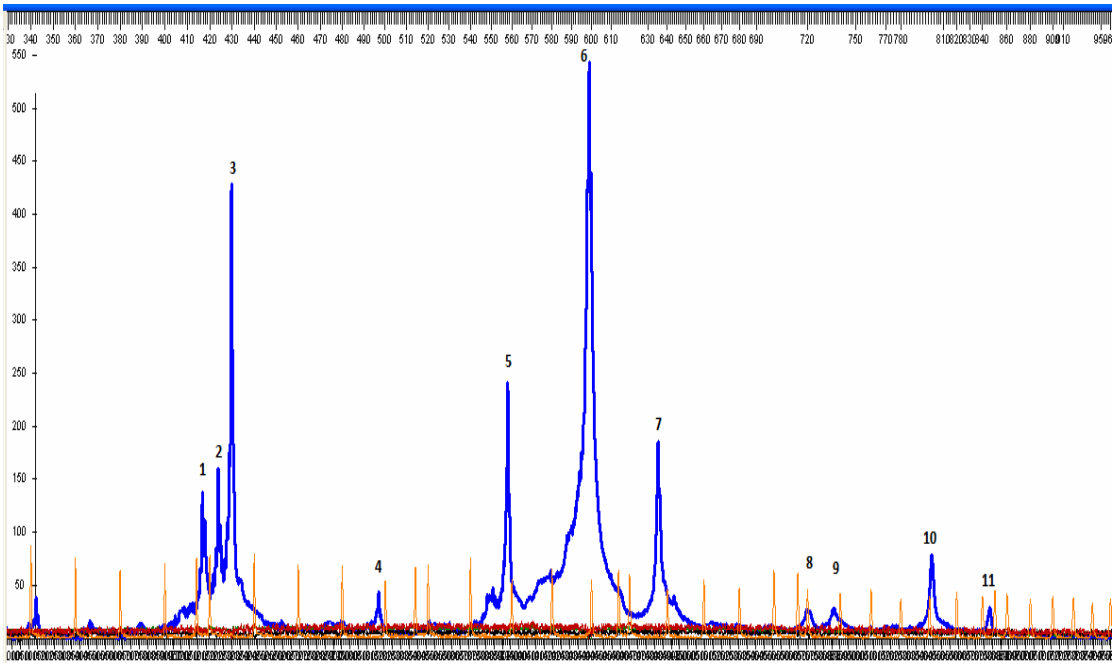

Figure 2.53: Mixture 4. The fragments produced using primer set mt-U1R and mt-U1F and a mix of 9 different species DNA at differing concentrations. These included *Canis mesomelas, Connochaetes taurinus, Damaliscus pyragargus, Damaliscus phillipsi, Alcelaphus bucelaphus, Ceratotherium simum, Capra hircus, Acinonyx jubatas* and *Felis catus.*

1 – 416bp fragment produced from *Acinonyx jubatas* and *Felis catus*

2 – 424bp fragment produced from *Acinonyx jubatas* and *Felis catus*

3 – 429bp fragment produced from *Canis mesomelas*

4 – 495bp fragment produced from *Capra hircus*

5 – 558bp fragment produced from *Ceratotherium simum*

6 – 598bp fragment produced from *Damaliscus pyragargus, Damaliscus phillipsi and Alcelaphus bucelaphus*

7 – 633bp fragment produced from *Connochaetes taurinus*

8 – 721bp fragment produced from *Felis catus*

9 – 735bp fragment produced from *Acinonyx jubatas*

10 – 802bp fragment produced from *Felis catus*

11 – 846bp fragment produced from *Capra hircus*

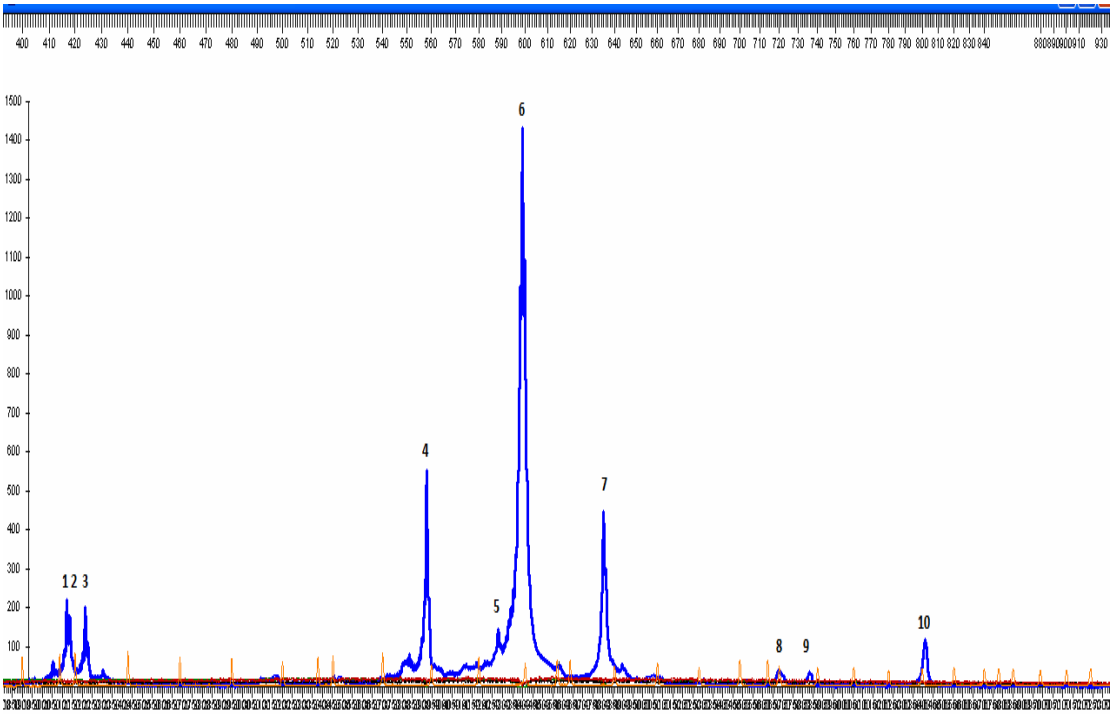

Figure 2.54: Mixture 5. The fragments produced using primer set mt-U1R and mt-U1F and a mix of 9 different species DNA at 1pg/µl. These included *Canis mesomelas, Aeryoeros melampus, Damaliscus pyragargus, Damaliscus phillipsi, Alcelaphus bucelaphus, Ceratotherium simum, Felis catus, Syncerus caffer* and *Acinonyx jubatas.*

1– 416bp fragment produced from *Felis catus* and *Acinonyx jubatas*

2 – 424bp fragment produced from *Felis catus* and *Acinonyx jubatas*

3 – 429bp fragment produced from *Canis mesomelas*

4 – 558bp fragment produced from *Ceratotherium simum*

5 – 594bp fragment produced from *Syncerus caffer*

6 – 598bp fragment produced from *Damaliscus pyragargus, Damaliscus phillipsi and Alcelaphus bucelaphus*

7 – 624bp fragment produced from *Aeryoeros melampus*

8 – 721bp fragment produced from *Felis catus*

9 – 735bp fragment produced from *Acinonyx jubatas*

10 – 802bp fragment produced from *Felis catus*

A specific mixture of samples of felid origin was tested in order to determine if the test can successfully determine between the closely relate species. The DNA concentration for all samples was 100 pg/µl. The *Panthera tigris* was included in the test despite it not being of African origin.

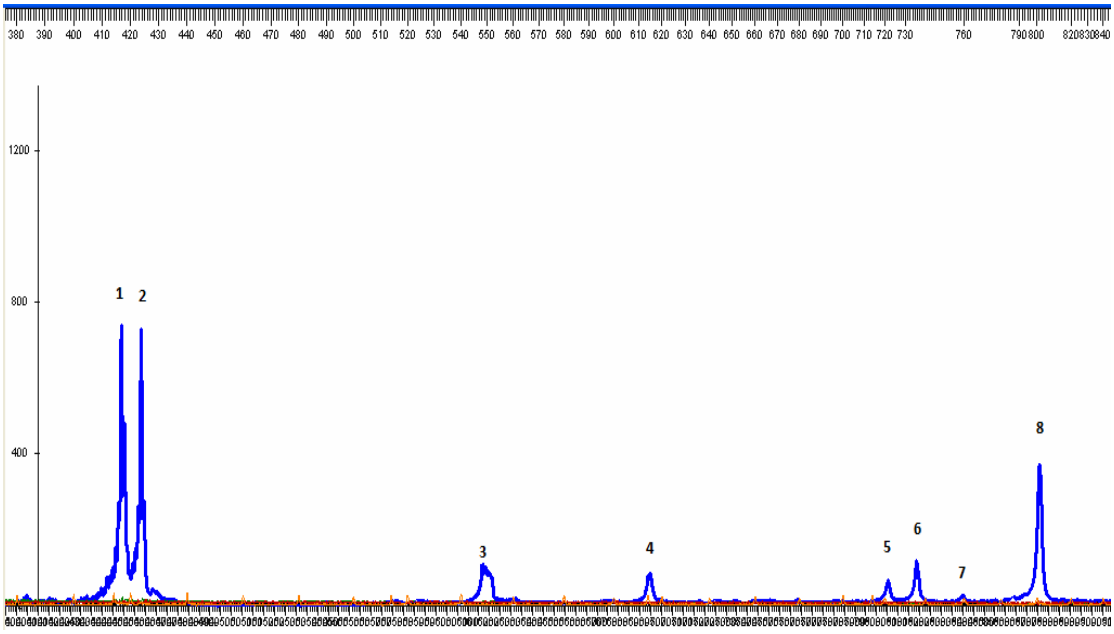

Figure 2.55: Mixture 6. The fragments produced using primer set mt-U1R and mt-U1F and a mix of 5 different feline species DNA at 100pg/µl. These included *Felis catus, Panthera leo, Acinonyx jubutas, Panthera tigris* and *Panthera pardus.*

1 – 416bp fragment produced from *Felis catus, Panthera leo, Acinonyx jubutas, Panthera tigris and Panthera pardus*

2 – 424bp fragment produced from *Felis catus, Panthera leo, Acinonyx jubutas, Panthera tigris and Panthera pardus*

3 – 550bp fragment produced from *Panthera leo*

4 – 615bp fragment produced from *Panthera tigris*

5 – 721bp fragment produced from *Felis catus*

6 – 735bp fragment produced from *Acinonyx jubutas*

7 – 758bp fragment produced from *Panthera leo*

8 – 802bp fragment produced from *Felis catus*

3.4 Data analysis

3.4.1 Comparison of results

Table 6: Comparison of results published by Nakamura *et al* (2009) and the results in this study.

Species	Fragment size (bp) Nakamura *et al.* (2009)	Fragment size (bp) This study	Difference (bp)
Dog	398.13	430	32
Pig	491.84	521	30
Cow	542.85	572	30
Sheep	800.44	495 / 821	21bp and an additional novel fragments.
Cat	695.16 / 774.80 / 854.10	416 / 424 / 721 / 802	25 and additional novel fragments.
Human	544.58 / 592.60	576 / 624	32 / 32
Chicken	597.28	622	25

3.4.2 BLAST results

A number of the animals produced fragments of the same size when capillary electrophoresis was performed. In order to look at a potential reason for this identical fragment size, sequencing was attempted. Due to the subsequent failure of this sequencing the sequences available on GenBank were used. BLASTn was used to compare the sequences of elephant and pig to the primer sequences in order to visualize the potential fragments amplified and compare the relative fragment sizes. The results show there should be a 8bp size difference in the fragments obtained.

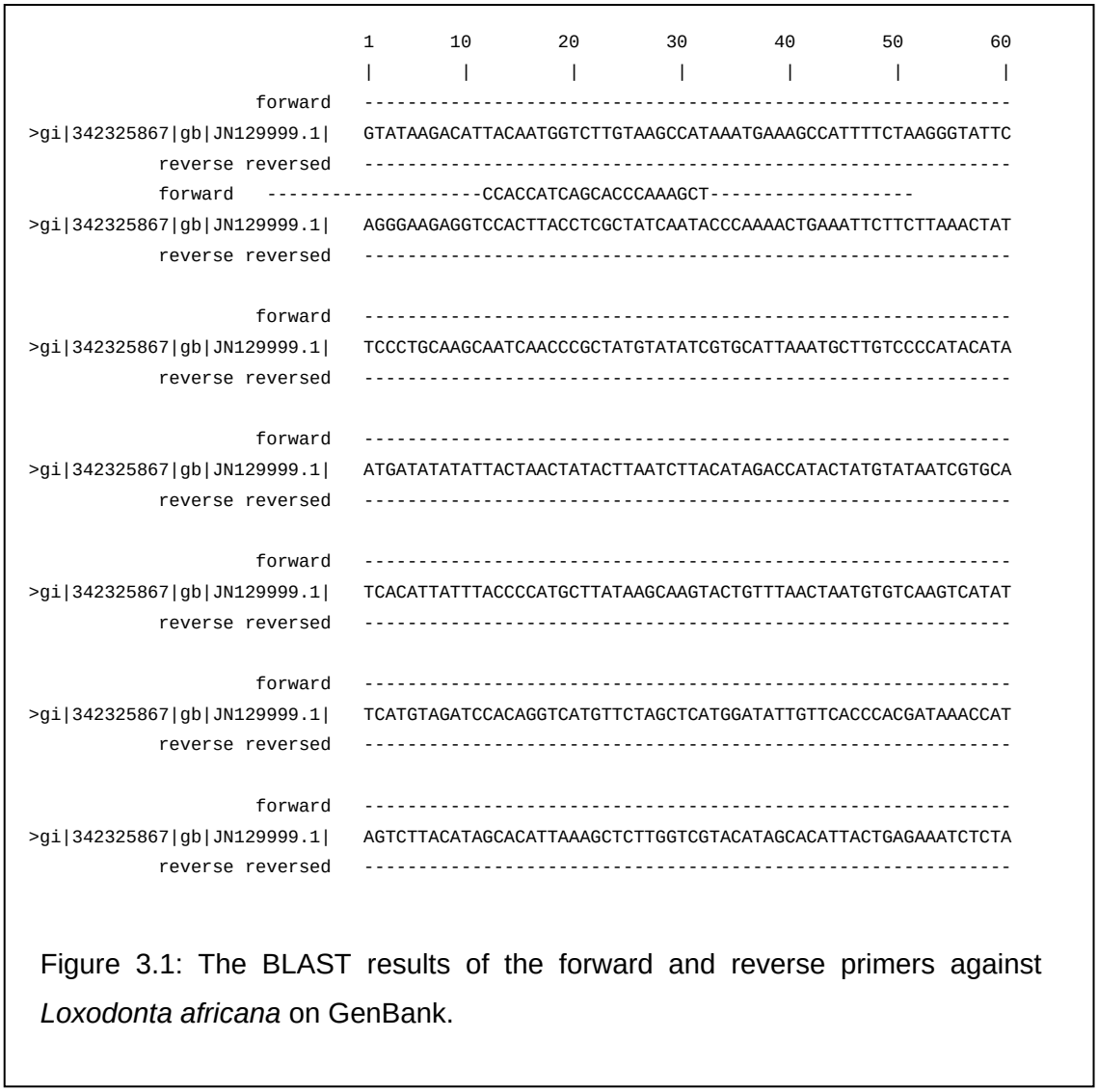

Figure 3.1: The BLAST results of the forward and reverse primers against *Loxodonta africana* on GenBank.

```
                            1        10        20        30        40        50        60
                            |        |         |         |         |         |         |
                    forward -------CCACCATCAGCACCCAAAGCT------------------------------
>gi|341819776|gb|JN031500.1| ACTAACTCCGCCATCAGCACCCAAAGCTGAAATTCTAACTAAATTATTCCCTGCAACCAA
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| AACAAGCATTCCATTCGTATGCAAACCAAAACGCCAAGTACTTAATTACTATCTTTAAAA
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| CAAAAAAACCCATAAAAATTGCGCACAAACATACAAATATGTGACCCCAAAAATTTAACC
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| ATTGAAAACCAAAAAATCTAATATACTATAACCCTATGTACGTCGTGCATTAATTGCTAG
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| TCCCCATGCATATAAGCATGTACATATTATTATTAATATTACATAGTACATATTATTATT
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| GATCGTACATAGCACATATCATGTCAAATAACTCCAGTCAACATGCATATCACCACCACT
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| AGATCACGAGCTTAACTACCATGCCGCGTGAAACCAGCAACCCGCTTGGCAGGGATCCCT
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| CTTCTCGCTCCGGGCCCATAAACCGTGGGGGTTTCTATTGATGAACTTTAACAGGCATCT
            reverse reversed ------------------------------------------------------------CT

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| GGTTCTTACTTCAGGGCCATCTCACCTAAAATCGCCCACTCTTTCCCCTTAAATAAGACA
            reverse reversed GGTTCTTACTTCAGGGCCA---------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| TCTCGATGGACTAATGACTAATCAGCCCATGCTCACACATAACTGAGGTTTCATACATTT
            reverse reversed ------------------------------------------------------------

                    forward ------------------------------------------------------------
>gi|341819776|gb|JN031500.1| GGTATTTTTTAATTTTTGGGGATGCTTAGACTCAGCCATGGCCGTCAAAGGCCCTAACAC
            reverse reversed ------------------------------------------------------------

                    forward --
>gi|341819776|gb|JN031500.1| AG
            reverse reversed --
```

Figure 3.2: The BLAST results of the forward and reverse primers against *Sus scrofa* on GenBank.

3.5 Case Studies

3.5.1 Case study 1

As can be seen in Figure 4.1, the capillary electrophoresis results showed the presence of only *Bos indicus* in the sample. This was verified by comparing this result to that of the control cattle sample (Figure 2.18) The GenBank BLAST results based on the cytochrome *b* sequencing performed in the laboratory using sequencing primers mcb398 and mcb395, show a 99% match to *Bos Indicus* (Figure 4.2)

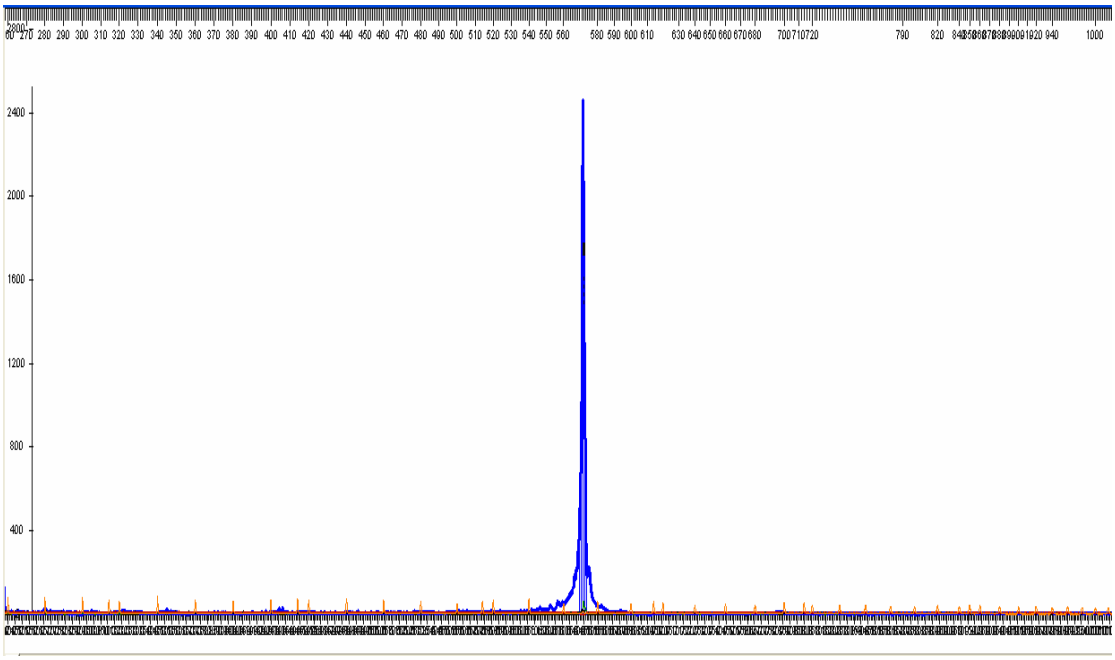

Figure 4.1: Results showing the amplification of *Bos Indicus* (domestic cattle) (572bp) from a meat patty of unknown origin.

gb|GU947021.1| Bos *indicus* isolate CLH_19 mitochondrion, complete genome
Length=16339

```
 Score = 518 bits (280), Expect = 1e-143
 Identities = 280/280 (100%), Gaps = 0/280 (0%)
 Strand=Plus/Plus

Query 1  GACAAAGCAACCCTTACCCGATTCTTCGCTTTCCATTTTATCCTTCCATTTATCATCATA 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15025 GACAAAGCAACCCTTACCCGATTCTTCGCTTTCCATTTTATCCTTCCATTTATCATCATA 15084

Query 61  GCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACCCAACAGGA 120
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15085 GCAATTGCCATAGTCCACCTACTATTCCTCCACGAAACAGGCTCCAACAACCCAACAGGA 15144

Query 121 ATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTA 180
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15145 ATTTCCTCAGACGTAGACAAAATCCCATTCCACCCCTACTATACCATTAAGGACATCTTA 15204

Query 181 GGGGCCCTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTC 240
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 15205 GGGGCCCTCTTACTAATTCTAGCTCTAATACTACTAGTACTATTCGCACCCGACCTCCTC 15264

Query 241 GGAGACCCAGATAACTACACCCCAGCCAATCCACTCAACA 280
    ||||||||||||||||||||||||||||||||||||||||
Sbjct 15265 GGAGACCCAGATAACTACACCCCAGCCAATCCACTCAACA 15304
```

Figure 4.2: BLAST results showing successful sequencing of the cytochrome *b* gene of *Bos Indicus* (domestic cattle) from a meat patty, performed in a separate confirmatory test.

3.5.2 Case study 2

Figures 4.3 and 4.4 show the amplification of a 637bp fragment. This fragment is comparable to the control sample run on the test (Figure 2.26). Figures 4.5 and 4.6 show the sequencing of the samples was successful and both samples returned BLAST results of a 97% match to eland.

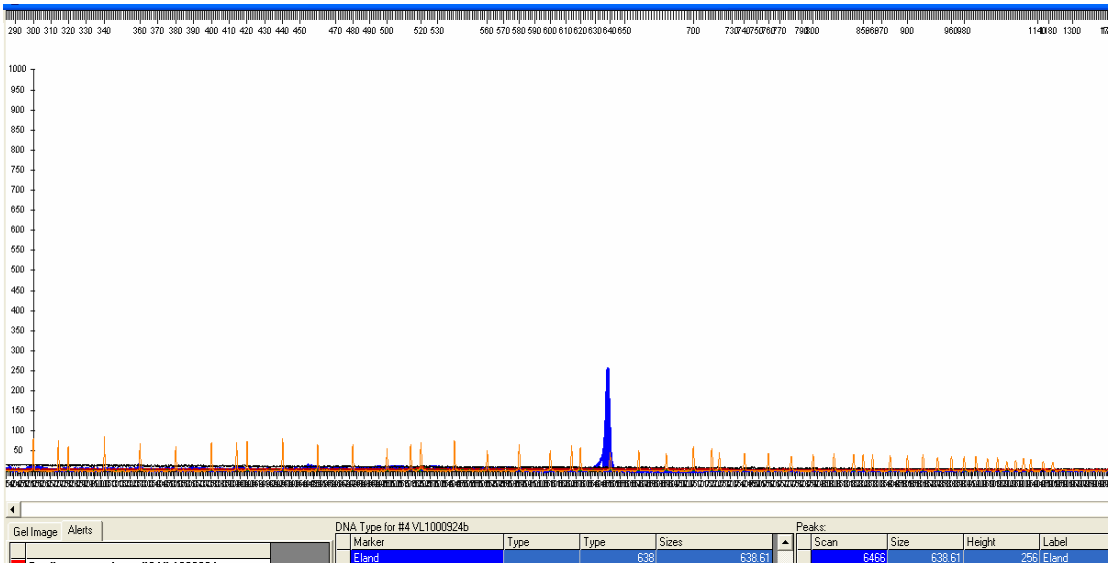

Figure 4.3: Results showing the amplification of *Tragelaphus oryx* (eland) (638bp) from a mince meat sample.

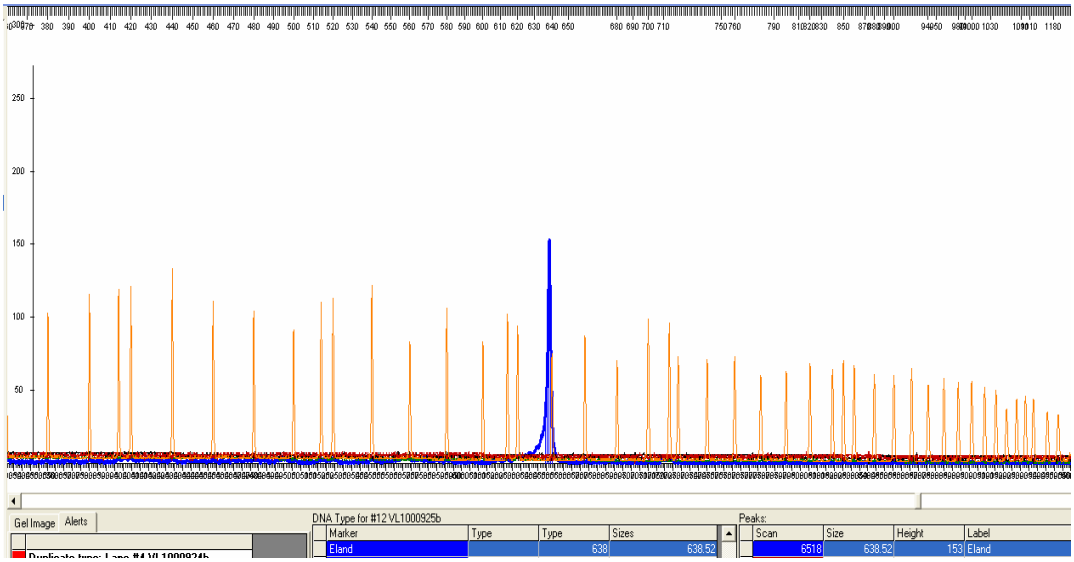

Figure 4.4: Results showing the amplification of *Tragelaphus oryx* (eland) (638bp) from a fillet sample.

gb|HQ122589.1| Tragelaphus oryx cytochrome *b* (cytb) gene, partial cds; mitochondrial

```
Length=517
 Score = 715 bits (387), Expect = 0.0
 Identities = 402/409 (98%), Gaps = 1/409 (0%)
 Strand=Plus/Minus

Query 1 TAATATGGGGAGGTGTGTTGAGTGGGTTTGCTGGGGTGTAGTTGTCAGGGTCTCCGAGGA 60
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 491 TAATATGGGGAGGTGTGTTGAGTGGGTTTGCTGGGGTGTAGTTGTCAGGGTCTCCGAGGA 432

Query 61 GGTCGGGTGCGAATAGTACTAGAAGTATTAGAGTTAGGACTAGTAATAGGGCGCCTAGAA 120
   |||||||||||||||||||||||||||||||||||| ||||| ||||||||||||||
Sbjct 431 GGTCGGGTGCGAATAGTACTAGAAGTATTAGAGTTAGGATTAGTAGTAGGGCGCCTAGAA 372

Query 121 TGTCCTTGATAGTGTGGTAAGGGTGGAATGGGATTTTGTCTATGTCTGATGAGATTCCTG 180
   |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct 371 TGTCCTTGATAGTGTGGTAAGGGTGGAATGGGATTTTGTCTGTGTCTGATGAGATTCCTG 312

Query 181 TTGGGTTGTTGGATCCTGTTTCGTGGAGGAATAGCAGGTGGACCATGGCTAGTGCTGCAA 240
   |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct 311 TTGGGTTGTTGGATCCTGTTTCGTGGAGGAATAGTAGGTGGACCATGGCTAGTGCTGCAA 252

Query 241 TAATAAATGGGAGGATGAAGTGGAAGGCGAAGAATCGGGTTAAGGTTGCTTTGTCTACTG 300
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 251 TAATAAATGGGAGGATGAAGTGGAAGGCGAAGAATCGGGTTAAGGTTGCTTTGTCTACTG 192

Query 301 AAAAGCCCCCTCAGATTCACTCAACTAGGCTGGTGCCGATATAAGGGATTGCTGATAGAA 360
   ||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct 191 AAAAGCCTCCTCAGATTCACTCAACTAGGCTGGTGCCGATATAAGGGATTGCTGATAGGA 132

Query 361 GGTTTGTGATAACTGTTGCTCCTCAGAATGATATTTGTCCCTCATGGTA 409
   |||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct 131 GGTTTGTGATAACTGTTGCTCCTCAGAATGATATTTG-CCCTCATGGTA 84
```

Figure 4.5: BLASTx results showing successful sequencing of the cytochrome *b* gene of *Tragelaphus oryx* (eland) from a mince meat sample.

gb|HQ122589.1| Tragelaphus oryx cytochrome *b* (cytb) gene, partial cds; mitochondrial

```
Length=517

 Score = 532 bits (288), Expect = 4e-148
 Identities = 307/316 (97%), Gaps = 2/316 (1%)
 Strand=Plus/Minus

Query 1 TTAGGACTAGT-GCTAGGGCGCCTAGAATGTCCTTGATATTGTGGTAAGGGTGGAATGGG 59
   |||||| |||| | |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 398 TTAGGATTAGTAG-TAGGGCGCCTAGAATGTCCTTGATAGTGTGGTAAGGGTGGAATGGG 340

Query 60 ATTTTGTCTATGTCTGATGAGATTCCTGTTGGGTTGTTGGATCCTGTTTCGTGGAGGAAT 119
   ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 339 ATTTTGTCTGTGTCTGATGAGATTCCTGTTGGGTTGTTGGATCCTGTTTCGTGGAGGAAT 280

Query 120 AGCAGGTGGACCATGGCTAGTGCTGCAATAATAAATGGGAGGATGAAGTGGAAGGCGAAG 179
   || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 279 AGTAGGTGGACCATGGCTAGTGCTGCAATAATAAATGGGAGGATGAAGTGGAAGGCGAAG 220

Query 180 AATCGGGTTAAGGTTGCTTTGTCTACTGAAAAGCCTCCTCAGATTCACTCAACTAGGCTG 239
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 219 AATCGGGTTAAGGTTGCTTTGTCTACTGAAAAGCCTCCTCAGATTCACTCAACTAGGCTG 160

Query 240 GTGCCGATATAAGGGATTGCTGATAGAAGGTTTGTGATAACTGTTGCTCCTCAGAATGAT 299
   |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct 159 GTGCCGATATAAGGGATTGCTGATAGGAGGTTTGTGATAACTGTTGCTCCTCAGAATGAT 100

Query 300 ATTTGTCGTCATGGTA 315
   ||||| | ||||||||
Sbjct 99 ATTTGCCCTCATGGTA 84
```

Figure 4.6: BLASTx results showing successful sequencing of *Tragelaphus oryx* (eland) from a fillet sample.

3.5.3 Case study 3

Figures 4.7 and 4.8 show the samples both produced peaks of 558bp, which matched the peak produced by the control sample of white rhinoceros (Figure 2.43).

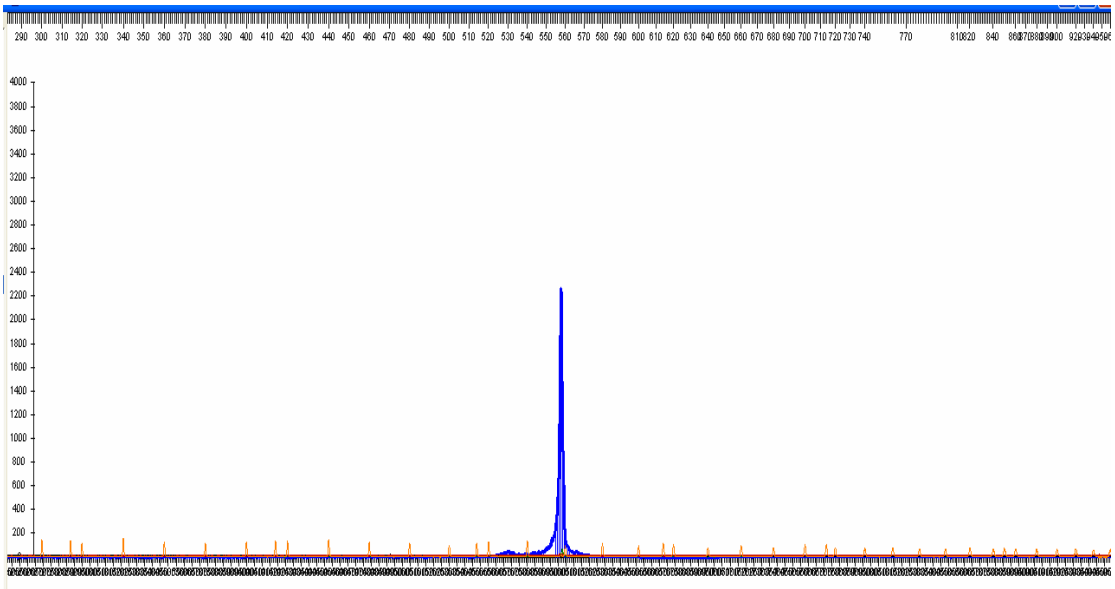

Figure 4.7: Results showing the amplification of *Ceratotherium simum* (558bp) from a forensic sample.

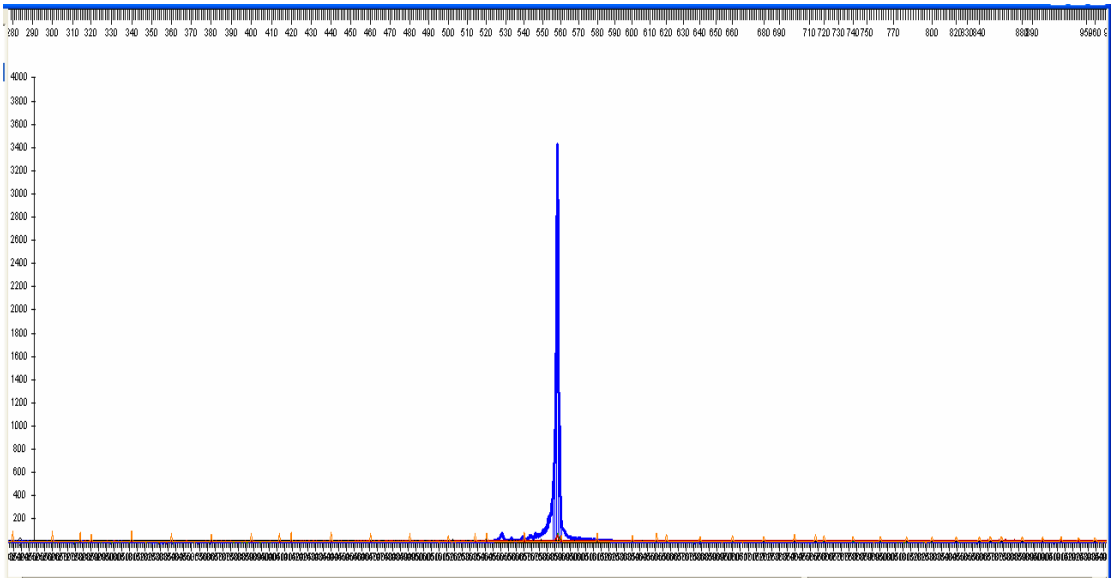

Figure 4.8: Results showing the amplification of *Ceratotherium simum* (558bp) from the second forensic sample.

CHAPTER 4: DISCUSSION

The demand for species identification of samples on a molecular level is increasing as people have realized the legal value of the results in court cases and criminal proceedings (Greg 2004; Gupta 2011; Linacre & Tobe 2011; Rosen & Smith 2010; Yip, Chau, Mak, Kwan 2007). Species identification based on the size variation of the mtDNA hypervariable regions among animals has been successfully used to discriminate between species (Nakamura *et al.* 2009) and in the study this method has been applied to several African wildlife species. In addition the test has a relatively low cost due to the limited number of primers required and is less labour intensive than the more traditional method of sequencing. An important limitation that can be encountered when dealing with African species identification is a lack of sequence data on GenBank. This can be avoided using this species identification system as long as a known control sample of the species in question is available.

4.1 Validation of the test – DNA quality and concentration

4.1.1 Sample extraction method and quality

The type of sample submitted to a laboratory can differ dependent on what species of animal the sample originates from, as well as the situation in which the sample is collected (ie whether it has been processed, degraded). The most common samples are tissue, hair roots and blood. The samples available for this study were largely that of salted tissue from a taxidermist as this allowed for reliable morphological identification. The extraction methods used on the different samples were specific to the sample types, some were extracted using standard extraction methods described previously, whilst others were extracted using adapted methods and specialized commercial kits. The different extraction methods yielded DNA extracts of differing quality and concentration (Table 2). Figures 2.1 to 2.4 show that amplification of

fragments was successful from a variety of different sample types. It also demonstrates that the type of extraction method used does not influence the speciation technique. This is important as it allows for a number of different sample types to be extracted and used. In the course of this investigation, a number of different sample types were looked at to determine the sensitivity of this species identification system (Table 3). Methods for the extraction of specific samples such as FTA slides, rhinoceros horn, teeth, swabs and elephant ivory were not within the scope of this dissertation. In all of these sample types, species identification was achieved regardless of sample condition, type and subsequent differing extraction methods. This is useful in the event that no other sample is available and the nuclear DNA tests have failed. A potential reason for the success of the test was due to the fact that it was based on mtDNA and this was present in much larger amounts in the samples when compared to nuclear DNA (Andreasson, Nilsson, Budowle, Lundberg & Allen 2006) .

4.1.2 DNA extract concentration

To investigate the specificity of this method further, we tested the DNA concentration limit of the test. Samples were originally used at a concentration of 10 ng/µl. The results showed an extremely high level of fluorescence (Figure 2.5), and therefore a series of dilutions were done in order to determine the optimal DNA concentration. Successful amplification of the fragment at a concentration of 1 pg/µl is shown in Figure 2.26. This concentration is lower than used by Nakamura *et al.* (2009) who described using a DNA concentration of 0.1 ng/µl. This concentration is relatively low, as routine fragment analysis is performed at a DNA concentration of 100 ng/µl. Once again this can be attributed to the high concentration of mtDNA in DNA samples (Andreasson *et al.* 2006)

In summary, very little sample material was needed in order to successfully amplify identifiable fragments. A large number of different sample types and

extraction methods were successfully used increasing the power of this test. This could be an extremely useful screening test for samples. The limitations however would require extra tests to be done if a verified sample of the original species was not available.

4.2 The application and success of the primer pairs and set up of a reference library of fragment lengths

A large reference library increases the chances that an unknown sample can be efficiently identified. The emphasis in this project was on African species as well as the common domestic species potentially found in the illegal wildlife trade.

4.2.1 Domestic species

Domestic species were initially tested as they were known to work and therefore were useful to optimize the primer function and PCR conditions. The PCR program described by Nakamura *et al.* (2009) was used and was successful. A number of species showed more than one fragment being amplified including sheep and goat, and cat. A 495bp fragment was amplified for both sheep and goat (Figure 2.12), however, there was an additional amplification of a specific fragment for each species, 821bp for sheep (Figure 2.15) and 846-848bp for goat (Figure 2.13).

Four separate fragments were amplified for the domestic cat (Figure 2.10). Further testing revealed that two of the fragments were constantly amplified for all feline species (424bp and 416bp) (Figure 2.8) while the other two fragments were specific for cat (Figure 2.9). These fragments showed a relatively high level of amplification when compared to the specific fragments amplified for each species. This could be due to the fact that the fragments are smaller in size than the species-specific fragments and the consumption of PCR reagents, otherwise known as the stoicheometry of the PCR process.

This was indicated by the higher fluorescence level constantly obtained. The amplification of numerous fragments could be due to two reasons. Firstly mtDNA heteroplasmy has been documented in a number of species and this could lead to two differing mtDNA hypervariable regions which could lead to the amplification of different sized fragments (Jae-Heup, Eizirik, O'Brien & Johnson 2001; Wilkinson & Chapman 1991). In the felid family, a number of peaks were expected as a length heteroplasmy has been found from the variable number of tandem repeats in the mtDNA-HV region (Lopez, Cevario & O'Brien 1996). The second possibility is the random co-amplification of nuclear mitochondrial pseudogenes. In the felid species, a nuclear mtDNA homologue, *Numt* has been described and resembles a potential nuclear pseudogene (Lopez *et al.* 1996). The differing fragments were effective at differentiating between the different species and therefore increased the specificity of the test. These results correlated with those presented by Nakamura *et al.* (2009) who reported up to five different fragments being produced when analyzing cat DNA. They attributed these fragment length differences to an 80bp repeat unit and the number of these present in the mtDNA. While this explanation is consistent with the 721bp and 802bp fragments detected by this study, it does not explain the two smaller fragments produced for all feline species.

4.2.2 Wildlife species

The results showed individual specific peaks for each species with the exception of elephant, pig, bontebok, blesbok and the red hartebeest. In the case of the blesbok and the bontebok, this could demonstrate the level to which the test is effective as these animals are sub-species and therefore this might have caused the similarity of fragment size (Essop, Harley & Baumgarten 1997). However this does not explain the similarity with the red hartebeest or the similarity of the elephant and pig. In investigating this similarity, the primer sequences were aligned using Geneious® (Drummond AJ 2010) and GenBank to both the pig and elephant mitochondrial DNA

sequences (Figures 3.1 and 3.2). The results showed that the forward primer bound to the sequence just outside of the D-loop control region in the tRNA pro region which was unexpected. This different target region could have affected the size of the fragments. The potential fragments amplified were compared and the difference between the two species was showed to be 8bp. The results did not reflect this difference. A potential factor that could have affected the fragment sizes reflected could be the migration of the fragments through the capillaries. The fragments differ in %GC and this can affect the charge of the molecule which can then affect the migration of the molecule (Yang, Bose & Hage 1996). In the scope of this study, these problems were not investigated further; however, the design of additional species-specific primers would be a solution to this. Alternatively if samples showed these results, further sequencing could be done to differentiate between the species. This limits the use of the test and makes it unacceptable as court evidence in forensic cases without the supporting sequence data. Following this, alignment of sequences from other species that produced similar peaks, namely the bontebok, blesbok, and red hartebeest, was attempted. Due to lack of sequence data on GenBank for these animals, this was not possible and therefore the reasons for the similarity between these species cannot be determined.

The results for the baboon showed a number of extraneous peaks being produced with the highest level of amplification of the 582bp fragment (Figure 2.46). The test was repeated using this species, however, the result was consistent. This was investigated and PCR conditions were changed in an attempt to reduce the amplification of these extra peaks. The DNA concentration was reduced to 0.1pg/µl, however, this resulted in unsuccessful amplification. The annealing temperature was raised to 58ºC, but once again the results showed no amplification of the baboon. Future research could look at designing more specific primers to more effectively identify the baboon species. The mitochondrion region has been suggested as an area where these specific primers could potentially be designed (Newman, Jolly & Rogers 2004).

A number of African wildlife species were tested and successfully identified. Results such as the differentiation between sable and roan were unexpected as these animals are closely related (Figure 2.25 and 2.26) (Matthee & Robinson 1999). In the case of white and black rhinoceros (Figure 2.43 and 2.44), and zebras and horses (Figure 2.50 and 2.17), there was a constant 1bp difference found.

In order to validate this, a number of samples were tested with the results positively confirming the constant 1bp difference over many different samples. It would be insightful to sequence the respective amplicons to identify/validate sequence/fragment size difference. This power of discrimination between these species was unexpected as previously discussed fragment length differences between individuals of a species were observed. The fact that these species are so closely related but are still distinguishable was an interesting observation.

4.2.3 Human and Avian primers

When working in a laboratory, human contamination (De Gruyter 1993) is a real possibility and therefore the primer pairs must effectively be able to detect human DNA in a sample. The primers designed by Nakamura *et al* (2009) were showed to effectively amplify a fragment for human; however, this was the same size as the fragment amplified for cattle. Due to this two further primers were designed in order to differentiate between the two species, mt-HV2R and mt-HV2F (Nakamura *et al.* 2009). These effectively allowed for the amplification of two different fragments in human but not in cattle. These fragments were the original fragment and another larger fragment. The results observed in this study differed slightly, with the single primer pair amplifying a 572bp fragment, while the two primer pairs amplified two completely different peaks (576bp and 624bp). This differs in that addition of the second primer pair appears to affect the size of the original fragment amplified. Despite this difference the primer pairs effectively distinguish human from cattle. This shows the potential of primers to be designed to effectively make a distinction

between other species with similar peaks such as the pig and elephant. This is an important feature of this method as human contamination is a problem in most laboratories and the low detection level of the test could monitor this very specifically.

The primers used for the avian species consisted of two differing forward primers and two reverse identical primers. This is due to potential mutation sites known to be present in the corresponding regions in the avian sequences. The avian primers were applied to chicken and successfully produced a specific fragment (Figure 2.48). Due to difficulty attaining samples, no other avian species were tested. It was important however to show that amplification of mammal species was not successful using these primers and that they are indeed avian specific (Figure 2.49). With more species, the avian primers could be tested further.

4.2.4 General Observations

Certain species with larger fragments (>600bp), did show fragment length differences among individuals within the same species of ± 2-3bp for example the wildebeest and goat. This could be as a consequence of electrophoresis differences when detecting the larger fragments or due to an analysis error when working with the larger size standards. Another possibility could be insertions/deletions in the mtDNA-HV (Angleby *et al.* ; Aspden, Pegg, Briskey & Sinclair 2006; Wilkinson *et al.* 1991) and should be taken into account when results are documented. Unfortunately as the sequencing of the fragments was unsuccessful, these differences could not be further investigated. By testing a large number of samples, one could determine for which species this is common and set up the fragment detection limits to incorporate this information.

4.3 Validation using known sample mixtures

A common problem in species identification can be that of mixed samples (Yip *et al.* 2007). If a sample contains more than one species, DNA identification can be difficult as sequencing would be inexact and potentially impossible due to mixed sequencing results. Due to the high level of sensitivity displayed by this method and the amplification of individual peaks, it was suspected that it would effectively be able to distinguish between species in the event of a mixed sample. In order to test this, sample mixtures were analyzed. The species and DNA concentrations included in these mixed samples were withheld and sample mixtures were prepared by a colleague so as to imitate a situation in which the species of the samples were completely unknown. The results were analyzed and then verified.

The sample mixtures were originally separated in terms of domestic and wildlife species and kept at a DNA concentration of 1pg/µl in order to monitor the amplification of the different species under the same conditions (Table 5). Different species were effectively identified from both the wildlife and domestic mixtures as shown in Figures 2.50 and 2.51 and, following this, the other sample mixtures were tested. These included both wildlife and domestic species and the concentrations of the DNA differed randomly to imitate a real potential field sample mixture. The individual species in the sample mixtures were all identified correctly. The species with the highest DNA concentrations showed the greatest fluorescence, however in the case of blesbok, bontebok and red hartebeest, the fragment amplified was constantly larger than the rest because the peaks were superimposed. In contrast, the feline species were hardly detectable despite having a higher concentration in the mixtures. This could be due to the larger fragment sizes of the felid species. This could be a limiting factor for this test as the detection of a feline species could be missed, however, if feline is suspected in the sample a higher concentration of DNA should be used which could potentially ensure detection. Another limitation of mixed samples of similar fragment size is that the peaks superimpose and cannot be differentiated. Sample mixture 6 consisted of just the feline species

and proved that differentiation between the important African feline species and the tiger was possible (Rosen *et al.* 2010). Sample mixtures are a useful application for forensic casework as samples often contain a mixture of species (Yip *et al.* 2007).

4.4 Sequencing

The sequencing of the similarly sized fragments would have been informative in determining the potential reason for this similarity. This however was not possible as the sequencing of the fragments was unsuccessful. The M13 tails attached to the primers were used as sequencing targets and specific M13 primers were used. For an unknown reason these were not successful and sequencing was not possible. Primer mtU1R was used as a potential sequencing primer, however, due to the fluorescence of the primers interfering with the sequencing reaction this was not possible. The reverse primer which was not labelled was used individually in order to attempt reverse sequencing, however this once again was unsuccessful.

4.5 Comparison of this study

The fragments produced by the primer pairs used in this study and those of Nakamura *et al.* (2009) did differ quite substantially (Table 6). For a number of species this difference was relatively constant between 30 and 32bp, however, for the species such as cat, the fragments were completely different in both size and number. The 30-32bp constant difference could be attributed to the fact that the primers had the M13 tails attached and these could have affected the size of the fragment being detected by the Genetic Analyzer. The difference in number of fragments for both cat and goat was not expected however is also potentially attributed to the difference in primer sequence due to the addition of the M13 tails.

4.6 Application of this species identification method

4.6.1 Case Study 1

Two separate tests were performed in order to determine the identification of the species present in the meat patty. The sequencing results detected the presence of *Bos indicus* in the sample (Figure 4.2). No other species was detected in the sample. The species identification test confirmed the sequencing results and detected only bovine in the sample (Figure 4.2). This case demonstrates the potential use of the species identification test in food quality control and commercial use. A number of products including fast food, meat mixtures (such as mince) and dog food can be monitored and this could be done quickly and inexpensively.

4.6.2 Case Study 2

The results of this case study illustrate the potential use of this test in the wildlife product trade sector. The effective identification of the meat as eland by both the species identification fragment analysis (Figure 4.3 and 4.4) and sequencing (Figure 4.5) is an example of the results that can be obtained in such a case. Products such as biltong and game meat can be screened cheaply using this method. This method could also aid in prosecuting cases of illegal hunting and poaching where sample is not morphologically identifiable.

4.6.3 Case Study 3

The illegal trafficking of rhinoceros horn has become increasingly common in South Africa during the last 3 years. Species identification of small pieces of rhinoceros sample has become an important tool in investigations.

The genotyping was unsuccessful and therefore species identification was done (Figure 4.7 and 4.8). The genotyping could have failed as a result of low levels and quality of DNA in the sample. The success of this test where the

genotyping has failed could be due to the fact that it was based on amplification of mtDNA rather than nuclear DNA. It could therefore be interpreted that both samples were taken from a rhinoceros, however due to the limitations of the method previously mentioned, one would need to do further testing to confirm this. This demonstrates the potential of this species identification method to be used in cases in which sample quality, type and condition has caused conventional methods to fail.

4.7 Limitations of the speciation method

An important fact to take into account is the limitations of this method. Firstly this method is only effective when morphologically verified sample of a species is available as reference. If a sample contains DNA from an animal that has not been tested and set up as a reference species, it would fail to be effectively identified. Alternatively the untested species could have the same size fragment as a previously tested but differing species. In this case the sample would be incorrectly identified. For this reason, the more reference samples tested on the method, the higher the discriminatory power becomes. The addition of sequencing of the fragments could be useful, however, it would increase the cost and time taken for the test.

CHAPTER 6: GENERAL CONCLUSIONS

Species identification based on the size variation of mtDNA hypervariable region in animals has proven to be an effective screening test, however, additional work would need to be done if it was to be used for official purposes. Thirty seven (37) different species were tested and the universal primer set could effectively distinguish between thirty two (32) of these species. Five results highlighted the limitation of the method as they returned similar fragment sizes for different species. BLAST analysis of the primers against reference sequences on GenBank revealed that for at least two of these species, elephant and pig, a fragment size difference was expected indicating that there is a potential for primers to be developed/re-designed that could differentiate between these similarities.

This method has an extremely low detection level and a high robustness for sample type and quality. A number of different sample types and extraction methods were used in this study and these successfully amplified fragments. In addition to this the DNA concentration used in the PCR reaction was lowered to 1 pg/µl, with positive results. This is important when dealing with samples where quality and content may be limited.

Further work could be done to sequence these fragments and this could therefore be used as a further test to confirm identity of a sample.

The method is also able to identify individual species in a mixed sample although this would need to be applied with caution as peaks could be superimposed causing failure of a species to be identified. This is however an important use of the test identifying different species in a mixed sample can be difficult.

One use for the test would be to rule out a sample as a certain species. This would be effective in ruling out the species applied to the test, however a control would need to be incorporated to monitor for PCR failure.

Nevertheless, this species identification method would be useful as an initial screening test for a number of African wildlife species as it is cost effective and fast allowing for a high throughput of samples.

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