REAL-TIME PCR DETECTION AND QUANTIFICATION OF ELEPHANTID DNA: SPECIES IDENTIFICATION FOR HIGHLY PROCESSED SAMPLES ASSOCIATED WITH THE IVORY TRADE

A thesis submitted to the committee on Graduate Studies in
partial fulfilment of the requirements for the Degree of Master of Science
in the Faculty of Arts and Science

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ABSTRACT

Real-time PCR Detection and Quantification of Elephantid DNA: Species Identification for Highly Processed Samples Associated with the Ivory Trade

Kristyne Michelle Wozney

The ivory industry is the single most serious threat to global elephant populations. A highly sensitive, species-specific real-time PCR assay has been developed to detect and quantify African elephant (*Loxodonta africana*), Asian elephant (*Elephas maximus*) and Woolly Mammoth (*Mammuthus primigenius*) mitochondrial DNA from highly processed samples involved in international ivory trade. This assay is especially useful for highly processed samples where there are no distinguishing phenotypic features to identify the species of origin. Using species-specific *Taq*man® probes targeting the mitochondrial cytochrome *b* region, the assay can be used to positively identify samples containing elephant or Woolly mammoth DNA faster and more cost-effectively than traditional sequencing methods. Furthermore, this assay provides a diagnostic result based on probe hybridization that eliminates ambiguities associated with traditional DNA sequence protocols involving low template DNA. The real-time method is highly sensitive, producing accurate and reproducible results in samples with as few as 100 copies of template DNA. This protocol can be applied to the enforcement of the Convention on the International Trade of Endangered Species (CITES), where positive identification of species in an illegally traded product is required by conservation-based enforcement officers in wildlife forensic cases.

Keywords: real-time PCR, *Taq*man®, CITES, species identification, mitochondrial DNA, cytochrome *b*, elephant, Woolly mammoth, wildlife forensics

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TABLE OF CONTENTS

Abstract ii

Acknowledgements iii

Table of Contents v

List of Tables vi

List of Figures vii

List of Appendices ix

Introduction 1

Methods 15

- Sample preparation and extraction 15
- Primer and probe design 16
- Development and optimization 20
- Analysis of specificity 23
- Reproducibility 23
- Evaluation of sensitivity on control DNA....................24

Results and Discussion 25

- Control development 25
- Analysis of specificity 28
- Reproducibility 32
- Evaluation of sensitivity on control DNA....................42
- Benefits of real-time PCR over DNA sequencing....................44

Conclusion 50

Literature Cited 62

LIST OF TABLES

Table 1. Total number of single nucleotide polymorphisms (SNPs) between *Taq*man® probe target species and other non-target elephant species..................................52

Table 2. Sequences and fluorescent labels for all primers and probes used in elephant species identification assays...52

Table 3. Primer and probe combinations for elephant species identification assays designed for two different portions of the mitochondrial cytochrome *b* region...........53

Table 4. Cross reactivity observed for elephant detection and species identification assays between other non-target elephant species...54

Table 5. The success rate for all replicates as well as means and variance within and between all three runs for elephant detection assays and three elephantid species.......55

Table 6. Summary of analysis of covariance (ANCOVA) results for nine elephant species-specific reactions...58

Table 7. Slopes and resulting reaction efficiencies and R^2 values for three runs of each elephant detection and species identification assay..59

Table 8. Results of real-time PCR amplification of a dilution series of DNA from three elephant species, using initial elephant screening assays..................................60

Table 9. Cost analysis of DNA sequencing and real-time PCR for species identification of 48 case samples...61

LIST OF FIGURES

Figure 1. Elephant ivory carvings seized by Canadian Wildlife Service (CWS) as a result of CITES enforcement..5

Figure 2. Elephant leather chessboard, motorcycle seat and gun case seized by CWS as a result of CITES enforcement..5

Figure 3. Elephant hair jewelry seized by CWS as a result of CITES enforcement.........6

Figure 4. A portion of a cytochrome *b* sequence obtained from amplification of DNA extracted from suspected leopard hide sample. Red circles indicate regions of ambiguity in base calling and/or spacing in the interpreted output from Sequence Analyser 3.0 (Amersham Biosciences Inc, Pittsburgh, Pennsylvania)..11

Figure 5. Sequence alignment showing regions for primer and probe design for fragment 1..18

Figure 6. Sequence alignment showing regions for primer and probe design for fragment 2..19

Figure 7. Amplification plot of dilution series of Woolly mammoth DNA amplified using species-specific reaction. The threshold cycle is indicated by the red line. Samples with 10^6 copies of template cross the threshold first around cycle 19, followed by 10^5, 10^4, 10^3, 10^2, 10 and 1 copy..33

Figure 8. Standard plot of threshold cycle and DNA template amount for a 6-fold dilution series ranging from 1 million to 1 copy of Woolly mammoth mitochondrial DNA, amplified in species-specific reaction. The resulting standard curve can be used for quantification of Woolly mammoth DNA in unknown samples.............................34

Figure 9. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount..35

Figure 10. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount..36

Figure 11. Box and whisker plots for three real-time PCR runs of Asian elephant species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount..37

Figure 12. Decision making tree and proposed workflow for elephant species determination using real-time PCR assays designed in this study..........................41

Figure 13. Dilution series of African elephant stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase. Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel...43

Figure 14. A portion of a Cytochrome *b* sequence from amplification and sequencing DNA extracted from a Woolly mammoth ivory sample. Red circles indicate regions of ambiguity in base calling in the interpreted output from Sequence Analyser 3.0 (Amersham Biosciences Inc, Pittsburgh, Pennsylvania)...................................46

LIST OF APPENDICES

Appendix I. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....69

Appendix II. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....70

Appendix III. Box and whisker plots for three real-time PCR runs of African elephant species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....71

Appendix IV. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....72

Appendix V. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....73

Appendix VI. Box and whisker plots for three real-time PCR runs of Woolly mammoth species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount....74

Appendix VII. a)Dilution series of African elephant stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
b). Dilution series of Asian elephant stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
c). Dilution series of Woolly mammoth stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase. Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel. 75

Appendix VIII a). Dilution series of Asian elephant stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
b). Dilution series of Woolly mammoth stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase. From left; Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel. 76

INTRODUCTION

One of the most important advances in the field of forensic science has been the use of genetic markers to identify the source of biological materials (Yao *et al.*, 2004 and Anderson *et al.*, 2005). Forensic analysis of human DNA is commonly used to solve crimes and identify human remains (Karlsson and Holmlund, 2007). The same technologies used in human forensics may also be applied to crimes involving the trade of endangered species. Specifically, the use of genetic markers to identify the species of origin of illegally traded plant and animal products can aid in the enforcement of laws designed to protect endangered species (Kyle and Wilson, 2006).

The Convention on the International Trade of Endangered Species (CITES) limits the international trade and movement of plant and animal species that are, or have the potential to be, threatened due to excessive commercial exploitation. In Canada, the Canadian Wildlife Service (CWS), a division of Environment Canada (EC), is primarily responsible for the enforcement and regulation of CITES. Species listed under the CITES agreement are organized into three groupings or Appendices based on the level of exploitation and enforcement required. Appendix I species are endangered as a result of international trade and overexploitation by humans. In general, international trade of Appendix I species is prohibited except in cases where the animal has been captive bred or artificially reproduced. Trade of Appendix I species requires permits from both the importing and exporting countries. Appendix II species are not endangered but could become so as a result of international trade, while Appendix III species are not endangered but are managed by the listing nation. Trade of Appendix II and III species requires appropriate permits from the exporting country (www.cites.ec.gc.ca).

Both African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephant species are currently listed under Appendix I of CITES as they are highly endangered as a result of exploitation (Comstock *et al.*, 2003, Stiles, 2004 and Gupta *et al.*, 2006). Despite CITES regulations, the illegal trade of endangered species is a highly lucrative business generating billions of dollars in revenue worldwide (Warchol *et al.*, 2003). For elephants there has been evidence of increasing illegal trade. Between August 2005 and August 2006 over 25 000 kilograms of ivory were seized worldwide, more than the combined total for the three years prior (Wasser *et al.* 2008). This works out to about 4000 elephants using an estimate of 6.6 kg of ivory/elephant (Wasser *et al.* 2008).

Researchers in Chad's Zakouma National Park estimate that in 2008 the elephant population in the park was reduced to only one thousand individuals from over three thousand in 2006 due to an increase in poaching (Eichenseher, 2008). The park also had an increase in human deaths due to poachers, with six deaths in 2007, more than had been recorded for the previous 16 years combined (Eichenseher, 2008). It has been estimated that 8% of the remaining African elephants are killed by poachers annually (Wasser *et al.* 2008). This death rate could mean the extinction of elephants in Africa by the year 2020 (Wasser *et al.* 2008).

Asian elephants are also at risk of extinction. The populations of Asian elephants in Vietnam, Cambodia and Laos declined by two thirds, from 6250 individuals in the late 1980's to only 1510 in 2000 (Stiles, 2004). The female Asian elephant does not have tusks, and as a result male elephants are being targeted by poachers (Gupta *et al.* 2006). The selective harvest of males causes highly disproportionate sex ratios and will impact a population's ability to recover due to the inability to find a mate (Gupta *et al.* 2006). The

ivory trade is recognized as the single most important cause of the decline in elephant populations worldwide (Stiles, 2004).

In addition to elephant ivory trade, there is also an interest in ivory from the extinct Woolly mammoth (*Mammuthus primigenus*). Mammoth carcasses may be found in the permafrost of Siberia and Alaska where these animals lived over 10 000 years ago and their tusks are often very well preserved (Burragato *et al.* 1998). This is the only extinct proboscidean species that consistently provides high quality, carvable ivory (Espinoza and Mann, 1991). The Woolly mammoth is not listed on CITES and therefore the commercial trade of mammoth ivory is not restricted. The trade in mammoth ivory has increased recently as global warming exposes frozen remains and gas and oil crews dig wells and ditches in the tundra of Russia (Kramer, 2008). Exports of mammoth ivory from Russia increased to 40 tonnes in 2007 from only 2 tonnes in 1989 (Kramer, 2008). There is evidence that poachers may be intentionally mislabelling elephant ivory as that of the extinct Woolly mammoth in order to avoid CITES regulations (Lister and Bahn, 2007). This fraudulent trade makes it increasingly important to accurately determine the species origin.

Traditionally, species identification was based on morphology and performed by taxonomists who specialized in a particular group of organisms. However, morphological-based identifications have significant limitations in many commercially traded products. For elephants tusks are removed from the animal of origin making it almost impossible to identify elephant species (Singh *et al.* 2006). Ivory is often carved into desirable shapes (Figure 1) which can be very small. In addition to ivory, elephant

leather and hair are also commonly traded. The leather and hair may be formed or dyed, making it increasingly difficult to identify species based on appearance (Figures 2 and 3).

The most common method to distinguish between elephant and mammoth ivory, is to measure the angle of the Schreger lines found on transverse sections of the tusk (Burragato *et al.* 1998, and Espinoza and Mann, 1991). These angles may also be used to distinguish between extant species (Singh *et al.* 2006, and Espinoza and Mann, 1991) but are not easily visible in processed tusks. Carvings can be very small and from different sections of the tusk with no visible Schreger lines (Burragato *et al.* 1998). Other methods for identification of ivory include isotopic analysis, ultraviolet fluorescence, inductively coupled plasma-atomic emission spectroscopy, inductively coupled plasma-mass spectroscopy (Singh, et al. 2006) and thermogravimetric analysis (Burragato *et al.* 1998). Ultraviolet fluorescence is limited in that it may only be used to distinguish a natural ivory product from a manufactured ivory substitute, but not to identify species of origin. The other methodologies listed are expensive and rely on interpretations of differences in the relative abundance of particular elements in the ivory of different species. All of the above methodologies are also specific to ivory. They are not applicable to other elephant products such as leather and hair that are common in international trade. As a result, there is a need for a more universal, reliable and cost-effective method of species identification of elephant products.

Figure 1. Elephant ivory carvings seized by the Canadian Wildlife Service (CWS) as a result of CITES enforcement.

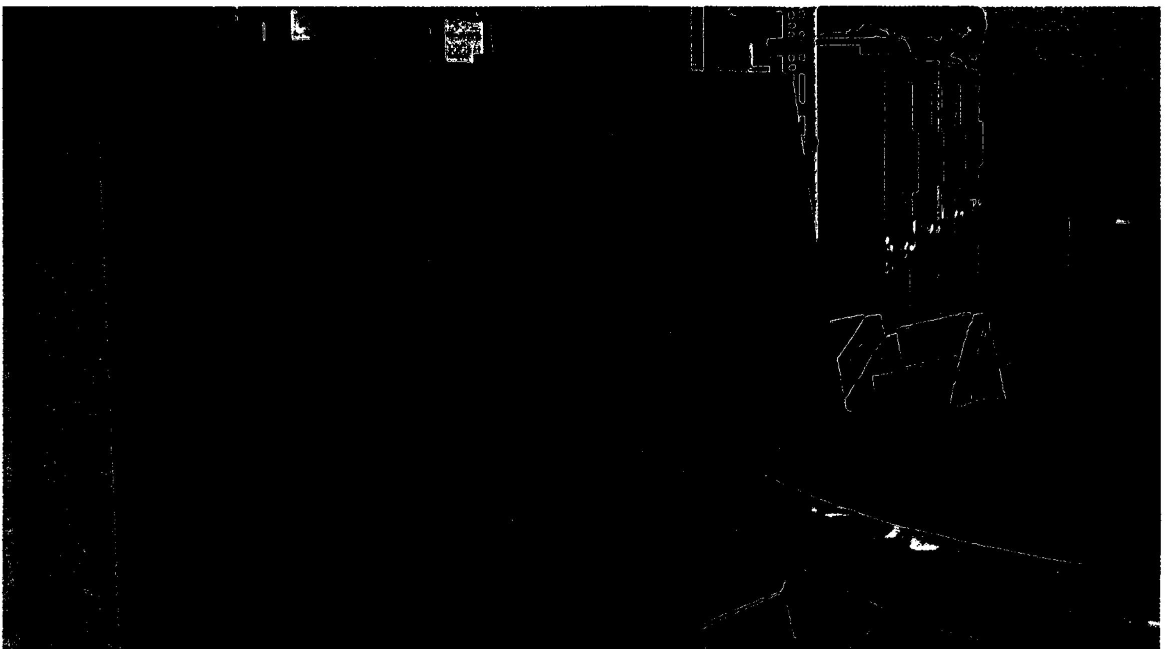

Figure 2. Elephant leather chessboard, motorcycle seat and gun case seized by CWS as a result of CITES enforcement.

Figure 3. Elephant hair jewelry seized by CWS as a result of CITES enforcement.

The importance of accurate species designations has led researchers to search for new methods of species identification. The most widely accepted method of species identification in forensic case samples is DNA sequencing. The portion of the genome that is sequenced depends on the species in question and may be found on the nuclear genome (Bellis *et al.* 2003), mitochondrial genome (Bataille *et al.* 1999, Parson *et al.* 2000, Hebert *et al.* 2003, and Bravi *et al.* 2004), ribosomal RNA (Bellis *et al.* 2003, and Kress *et al.* 2005), or the chloroplast genome of plants (Kress *et al.* 2005). The marker chosen must be variable between closely related species but stable within individuals of the same species (Hebert *et al.* 2004). For many taxa, markers found on the mitochondrial genome are commonly used in forensic species identification (Bellis *et al.* 2003, Wan and Fang, 2003, Dawnay *et al.* 2007, Karlsson *et al.* 2007).

The mitochondria of a cell contain a circular DNA molecule that is independent of the nuclear genome (Butler and Levin, 1998, Budowle *et al.* 2003, Pakendorf and Stoneking, 2005 and Snustad and Simmons, 2006). Mitochondrial DNA (mtDNA) is maternally inherited in mammals and does not recombine (Butler and Levin, 1998, Baasner *et al.*, 1998, Tully *et al.* 2001, Budowle *et al.* 2003, and Pakendorf and Stoneking, 2005), as a result an individual's mtDNA will be identical to that of their maternal parent (Butler and Levin, 1998) and all maternally related individuals.

Exceptions to the clonal inheritance of maternal mtDNA haplotypes have been documented (Butler and Levin, 1998). The low fidelity of mtDNA polymerase and a lack of repair mechanism during replication may result in mutations or heteroplasmy (Butler and Levin, 1998). Heteroplasmy refers to incidences where more than one sequence of mtDNA exists in the same individual (Butler and Levin, 1998, Melton and Nelson, 2001 and Budowle *et al.* 2003). Heteroplasmy is common and well documented in the hypervariable regions of the human mtDNA genome (Butler and Levin, 1998). Heteroplasmy may lead to inconclusive results if different sequences are observed in two samples under comparison. There may also be very rare cases of paternal inheritance or recombination between maternal and paternal mtDNA; however the rate at which this may occur and the mechanisms for recombination are both highly debated (Budowle *et al.* 2003). Paternal inheritance will have no impact on species identification except in cases of hybridisation where the paternal species will be identified instead of the maternal species.

Species identification has been performed using a number of different regions of the mitochondrial genome. One of the most common is the cytochrome *b* region

(Bataille *et al.* 1999, Parson *et al.* 2000, and Bravi *et al.* 2004). This marker is highly conserved within a species, but variable between species (Johns and Avise, 1998, and Parson *et al.* 2000). Many species representing all five major vertebrate groups exhibited over 99% intraspecific similarity in cytochrome b sequences (Parson *et al.* 2000). A study of 18 wild and domestic species of vertebrates endemic to Taiwan reported intraspecific sequence diversity ranges of 0.25-2.74% versus interspecific divergences of 5.97 to 34.83% (Hsieh *et al.* 2001). Sequencing of the cytochrome *b* region has been used to identify a number of species including big cats (Hsieh *et al.* 2001, Wan and Fang, 2003, Verma *et al.* 2003), sturgeon (Ludwig, 2008), aquiline eagles (Helbig *et al.*, 2005), Pecora (*Guha et al.* 2006), as well as to distinguish 16 species of carnivores of the Iberian Peninsula (Fernandes *et al.* 2008).

Sequencing of cytochrome *b* has also been well established for use in the species identification of forensic samples (Bataille *et al.* 1999, Parson *et al.* 2000, Branicki *et al.* 2003 and Bravi *et al.* 2004). DNA from various tissues including blood, saliva, soft tissues, animal hairs and bristles, bird feathers, dried shed skin, old bones and heated and processed meat were used to validate the use of this marker for forensic purposes (Branicki et al. 2003). In addition, cytochrome *b* has been applied to forensic case work and has been used to identify species from case samples of meat (An *et al.* 2007), blood and hair (Nakaki *et al.* 2007), as well as from bones seized from traditional Chinese medicine traders (Wetton *et el.* 2002), ivory from elephants, and horns from three species of rhinocerous (Bollongino *et al.* 2006).

DNA is a highly stable molecule compared to RNA and proteins but can degrade as a result of environmental conditions such as UV light, heat, genotoxins from some

bacteria and acidic environments (Mitchell *et al.* 2005). Mitochondrial DNA is less sensitive to environmental conditions and does not break down as quickly as nuclear DNA (Butler and Levin, 1998). This is likely due to the circular structure of the molecule (Murray *et al.* 2007). Furthermore, fragments of mtDNA persist in tissue such as bone, teeth and hair and consequently DNA can be extracted from very old or ancient samples (Yang and Speller, 2006). Mitocondrial DNA is also present in much higher copy numbers in a cell than nuclear DNA (Tully *et al.* 2001). A single mitochondrion contains 2-10 copies of the mitochondrial genome, with tens to thousands of mitochondria present per cell (Butler and Levin, 1998, Budowle *et al.* 2003, and Pakendorf and Stoneking, 2005). This makes mitochondrial markers desirable as they are more likely to be present and identifiable in a sample than single copy nuclear genomes (Butler and Levin, 1998, and Branicki *et al.* 2003). This is particularly important in forensic samples that are not properly preserved and like ivory specimens, have been processed in some way (Branicki *et al.* 2003). For many forensic samples, nuclear DNA marker analysis is impossible due to degradation and as a result mtDNA analysis is becoming increasingly important in the field (Butler and Levin, 1998, Bataille *et al.* 1999, Bar *et al.* 2000, Parson *et al.* 2000 Budowle *et al.* 2003, Branicki *et al.* 2003).

The characteristics that make mtDNA analysis valuable in forensics also result in the need for stringent protocols. The high copy number and stability of the mtDNA molecule increases the chances of contamination during processing (Bar *et al.* 2000). Contamination must be monitored using reagent blanks and negative controls. Both an extraction blank and a polymerase chain reaction (PCR) negative should be processed (Bar *et al.* 2000). A positive control should be inserted at the PCR stage and processed

through to sequencing (Tully *et al.*, 2001). If any negative control exhibits the presence of DNA or the positive controls yield a sequence that is the same as that obtained from the evidence, the results are rejected and the analysis repeated (Bar *et al.,* 2000). The use of appropriate standards and controls are important in exhibiting that both the lab and tests performed are reliable (Carracedo *et al.*, 1997). For human forensic testing it is desirable to duplicate mtDNA sequencing procedures (Butler and Levin, 1998 and Bar *et al.*, 2000). The potential for contamination of human samples is greater because samples are collected and processed by other humans, thus extra precautions must be taken. Despite the increased potential for contamination, incidences of heteroplasmy and possible paternal leakage and genome recombination, PCR based mtDNA typing by automated sequencing has been validated as a robust and reliable means of forensic identification (Butler and Levin, 1998, Bar *et al.,* 2000, Parson *et al.,* 2000).

While DNA sequencing is the most common assay for species identification of forensic samples, the process requires several steps and is relatively expensive (Ludwig, 2008, and Fernandes *et al.* 2008). In order to determine a sequence, one must perform a series of amplifications, purifications and finally a number of analysis steps to determine the species of origin. While it may be possible to amplify a region of interest for species identification, it is not always possible to obtain a sequence of high enough quality from low template samples to be admitted as evidence in court. For example, samples from rugs suspected of being made from leopard hide were unidentifiable using DNA sequencing (Wozney, unpublished data). Samples showed amplification of the cytochrome *b* region on an agarose gel, but the highest quality sequence was a quality of only 89% (Figure 4) and thus un-interpretable. A small portion of the NADH region was

amplified and sequenced successfully. Further analysis of the resulting NADH sequence identified the samples as having originated from a large cat species. However this region did not exhibit enough interspecific variation to identify the species of origin and as a result of inconclusive evidence this case was never prosecuted.

Figure 4. A portion of a cytochrome *b* sequence obtained from amplification of DNA extracted from a suspected leopard hide sample (Wozney, unpublished). Red circles indicate regions of ambiguity in base calling and/or spacing in the interpreted output from Sequence Analyser 3.0 (Amersham Biosciences Inc, Pittsburgh, Pennsylvania).

DNA sequencing may also be affected by complex mixtures and by inhibitors found in many forensic samples (Wetton *et al.*, 2002). Often, poor sequence quality and ambiguous base calling make it difficult to determine species in poor quality or low template samples. As a result, many cases of illegal importing or exporting are not prosecuted due to the lack of evidence that a product truly derives from a CITES-listed species (Wozney unpublished data). In general it is difficult to obtain reliable evidence to assist in the prosecution of individuals of illegally trading endangered species (An *et al.* 2007, and Bollongino *et al.* 2003). This is especially true in cases where there are

very small pieces of evidence and/or processing prevents morphological identification (Bollongino *et al.* 2003). Positive identification of the species of origin should be obtained before prosecution is considered (Wetton *et al.* 2002) and enforcement is often hampered by this lack of evidence (Singh *et al.* 2006).

Real-time PCR is a sensitive method that can be used to positively identify the presence of specific DNA fragments at very low quantities (McCartney *et al.*, 2003). Real-time PCR measures the rate of amplification throughout the cycles as opposed to traditional PCR which requires the use of some type of end point analysis. Real-time PCR requires the binding of a species-specific *Taq*man® (®Applied Biosystems, Grove City, California) probe to produce a fluorescent signal as well as primers to locate the markers. Real-time technology utilizes the 5' exonuclease activity of *Taq* polymerase, combined with Fluorescent Resonant Energy Transfer (FRET) to detect PCR amplification. The 5' exonuclease activity of the *Taq* polymerase removes any DNA that is downstream and may impede synthesis of a new strand (Sequence Detection Systems, Chemistry Guide for ABI Prism 7900, Applied Biosystems, 2003). An oligonucleotide or probe is designed to anneal to the target sequence between the forward and reverse primers. The probe contains a high energy reporter dye on the 5' end and a low energy dye or quencher on the 3' end. When the dyes are in close proximity there is a transfer of energy from high to low dye. When the polymerase reaches the probe, which has annealed in the pathway of the enzyme, the 5' exonuclease activity cleaves the probe causing the energy transfer from reporter to quencher to stop (McCartney *et al.*, 2003). This results in an increase in fluorescent emissions that is detected by the sequence detection instrument. Emission of fluorescence positively identifies the presence of DNA

from a particular species. The intensity of fluorescence is proportional to the amount of amplicon created. Amplification detection is measured using the value of the cycle threshold (C_t). This is the point at which the reaction is at the mid-exponential phase or when a significant increase in fluorescence is observed above the baseline fluorescence. The C_t is inversely proportional to DNA quantity. A higher amount of template will result in a lower C_t value. Through plotting the observed fluorescence or C_t against the quantity of starting template in control samples, real-time PCR can also be used to quantify amount of DNA in unknown samples (McCartney *et al.*, 2003).

Real-time PCR has been used to positively identify small quantities of mtDNA from human peripheral blood and subcutaneous fat cells (Gahan *et al.*, 2001), to quantify mtDNA in forensic samples (von Wurmb-Schwark *et al.* 2002), to quantify both mtDNA and nuclear DNA in forensic samples and ancient human bone (Alonso *et al.* 2003), as well as for species identification of tiger from blood samples (Wetton *et al.* 2002). Real-time species-specific assays have been developed for identification of a number of different animal and bacterial species as well as for identification of viral strains. Four serotypes of hantavirus are distinguishable with a real-time PCR assay (Aitichou *et al.*, 2005) as are five subspecies of *Clavibacter michiganensis*, a plant pathogen (Bach *et al.*, 2003). Real-time PCR has similarily been used to detect and quantify porcine, bovine, lamb, turkey, chicken and ostrich in complex samples (Lopez-Andreo *et al.* 2005).

There are many benefits to real-time PCR. As mentioned above, real-time PCR measures the rate of amplification as opposed to total amplification over a designated period of time. This eliminates the need for end-point separation analysis using an agarose or acrylamide gels, thus reducing the chances of contamination (von Wurmb-

Schwark *et al.*, 2002) as well as reducing overall costs. The assay works very well with small DNA fragments, making it very useful for degraded samples. For example, in highly degraded ancient bone samples, a 133 base pair fragment of mtDNA was amplified but not a 287 base pair fragment (Alonso *et al.* 2003). Real-time PCR has been shown to be more sensitive than traditional PCR combined with endpoint analysis (McCartney *et al.*, 2003), and some real-time assays may detect single copy quantities of a gene (Alonso *et al.* 2003). In addition, real-time assays eliminate difficulties associated with poor quality sequencing results. The combination of species-specific probes and primer specificity make this type of assay more definitive, without the problems of inconsistent, imperfect or ambiguous results. Probe specificity allows for the detection of DNA from a target species even in the presence of DNA from other species. Real-time PCR assays produce reliable and consistent results for forensic purposes (Gahan *et al.,* 2001, and von Wurmb-Schwark *et al.*, 2002).

We have developed a real-time assay that targets a 103 base pair fragment of the cytochrome *b* gene on the mitochondrial genome. The assay has been designed to positively identify and distinguish among African elephant (*Loxodonta africana*), Asian elephant (*Elephas maximus*) and Woolly mammoth (*Mammuthus primigenius*). The protocol involves an initial screening and quantification of elephantid mtDNA using a *Taq*man® probe designed to anneal to the DNA of all three species. Following the initial screening, species may be distinguished through the use of species-specific *Taq*man® probes. This assay may be used to identify species of origin in processed samples related to CITES enforcement.

METHODS

Sample Preparation and Extraction

A blood sample from an African elephant and toenail sample from an Asian elephant were obtained for use as positive controls for all experiments. Control samples of other exotic animals such as camel, river hippopotamus, white rhinoceros and Indian rhinoceros, water buffalo, warthog and bovine were also obtained to ensure species from the same geographic regions did not show false positive results. Bones or horns from these animals may also be exported and may be mistaken as ivory especially if carved. All control samples were acquired from The Toronto Zoo, Ontario, Canada with the exception of the Asian elephant toenail, which was obtained from the Calgary Zoo in Alberta. A forest elephant (*Loxodonta cyclotis*) control sample could not be obtained and this form of African elephant was therefore not included in the development of this assay. The taxonomic status of the forest elephant is uncertain (Roca *et al.* 2001 and Eggert *et al.* 2002). This smaller form of African elephant may be a separate species or a sub-species of the African elephant. At the present time CITES does not recognize the forest elephant as a separate species and this morphotype is considered an African elephant (www.cites.ec.gc.ca). For the Woolly mammoth a control sample of ivory was provided by the Canadian Wildlife Service, Burlington, Ontario and a fossilized sample was also provided by the Canadian Museum of Nature, Ottawa, Ontario.

Two Woolly mammoth ivory samples were processed by drilling or grinding in liquid nitrogen to break up ivory into small particles for proper cell lysis. The resulting powder was incubated overnight in 0.5M EDTA (Invitrogen, Carlsbad, California) to

remove calcium. Following overnight incubation at 37°C, the EDTA was removed and extraction proceeded with all other samples using the following protocol: Samples were prepared in 500μl of lysis buffer (4M urea, 0.2M NaCl, 0.5% n-lauroyl sarcosine, 10mM 1,2-cyclohexanediaminetetraacetic acid, 0.1M Tris-HCl, pH 8.0). Each sample was incubated with 10U of Proteinase K (Roche Diagnostics Corporation, Indianapolis, Indiana) at 65°C for 2 hours. Samples were incubated with another 10 U of proteinase K at 37°C overnight. Samples were then extracted using a Qiagen manual extraction following the protocol for animal tissues in the DNeasy Tissue Handbook (Qiagen, Valencia, California). DNA was eluted from the Qiagen column by adding 50μl of 65°C $TE_{0.1}$ (10mM Tris, 0.1mM EDTA).

Primer and Probe Design

All primers and probes were designed using the software Primer Express version 2.0. (Applied Biosystems, Grove City, California). Primers and probes were initially designed to distinguish between the two extant species of elephants. For the original fragment (fragment 1), primers and probes were designed from control and Genbank sequences which were amplified using the primers GLUDG-L and CB2-H (Palumbi, 1996). Sequences of African elephant, Asian elephant, Woolly mammoth, Pygmy (forest) elephant, dugong, rhinoceros, hippopotamus and human were aligned using Bioedit 6.0. (Hall, 1999). Universal elephant probes and primers and species-specific probes were designed for Asian and African elephants. The primers amplify a 145 base pair fragment of the cytochrome *b* gene initiating at base pair 14433 of the mitochondrial genome of the African elephant. The universal probe and primer combinations were designed to target

regions of variability between elephants and other mammals, but not between the two elephant species. The universal probe was designed as an initial screening test for elephant in unknown samples before species identification. For species identification, species-specific probes were designed to anneal in regions that maximized the number of single nucleotide polymorphisms (SNPs) between the two elephant species. Further research revealed illegal ivory trade may also involve the extinct Woolly mammoth (Lister and Bahn, 2007) and a species-specific probe was subsequently designed for this species.

In the interest of making the assays highly specific to each species, an additional set of both universal and species-specific primers and probes were designed upstream from the original set at base pair 14983 on the African elephant mitochondrial genome. The probes for this additional region (fragment 2) incorporated a higher number of SNPs between our target species. The sequence alignments for both cytochrome *b* fragments including locations of primers and probes are shown in Figures 5 and 6. The number of SNPs between each species-specific probe and the other two species are summarized in Table 1.

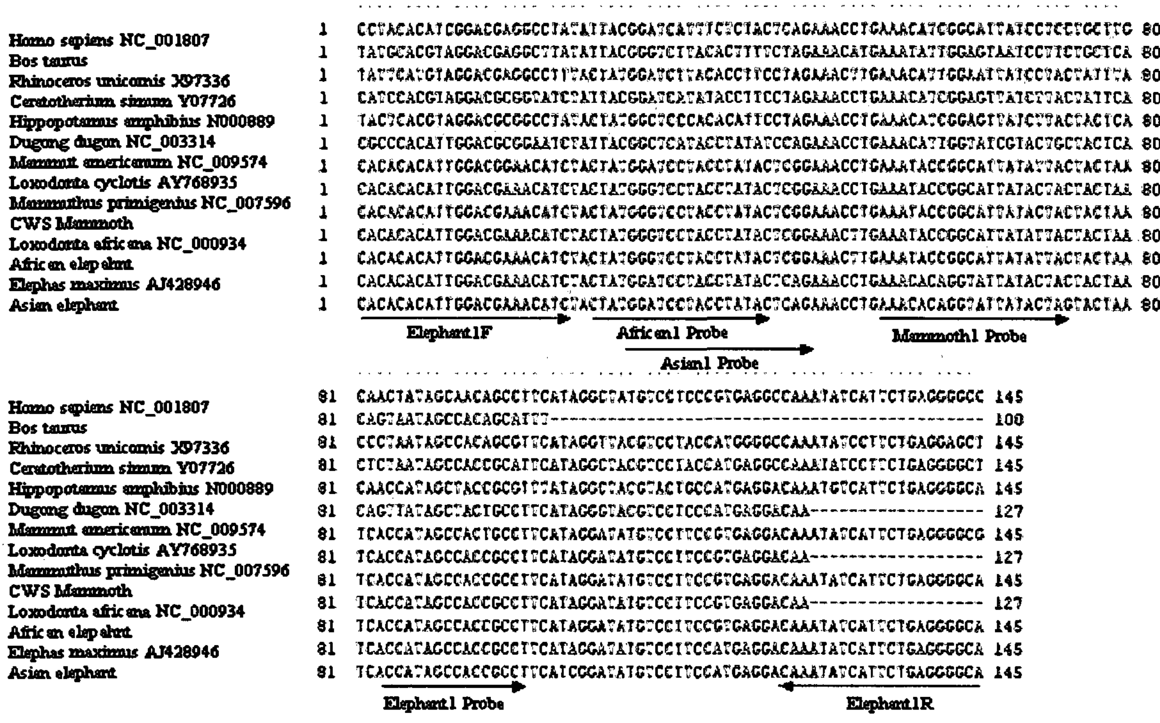

Figure 5. Sequence alignment of elephant species and other mammals for primer and probe design for fragment 1.

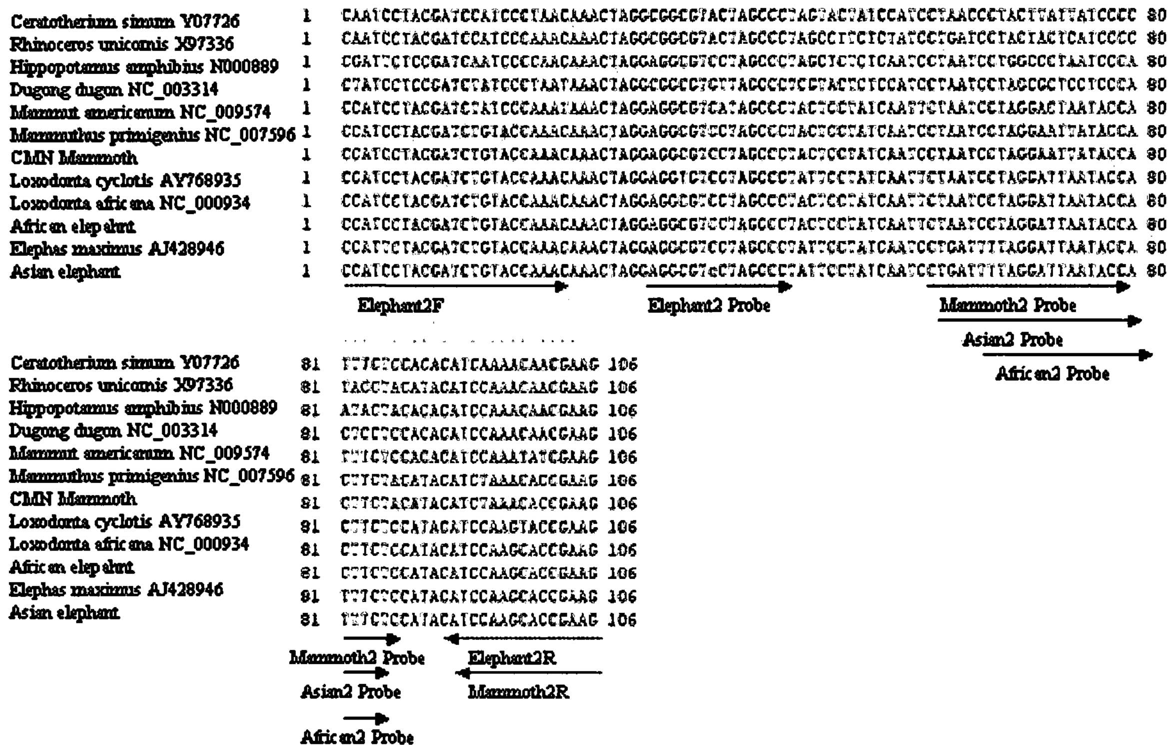

Figure 6. Sequence alignment of elephant species and other mammals for primer and probe design for fragment 2.

Primer and probe sequences are listed in Table 2. All primer and probe sets were designed to work at the same cycling conditions to allow for mixing of the same primers with different probes. Primer and probe combinations for each assay are summarized in Table 3. Universal primers are used in all species-specific assays except for Woolly mammoth fragment 2 where a species-specific reverse primer is used as well as a species-specific probe. Primers and probes were obtained from Applied Biosystems (Streetsville, Ontario). Probes contained a fluorescent dye on the 5' end and non-fluorescent quencher and minor groove binder on the 3' end. Standard protocols for real-time PCR suggest

that the melting temperature of the probe should be 10°C above that of the primers. The addition of a minor groove binder (MGB) will increase the melting temperature of the probe without increasing the length of the probe (Kutyavin *et al.* 2000).

Development and Optimization

A standard curve of mtDNA was developed for fragment 1 through amplification and quantification of a DNA fragment containing the target region for the designed primer and probes sets (Alonso *et al.*, 2003). Control samples for each species were amplified in a 50µl reaction containing 1x PCR buffer, 0.2mM of each DNTP, 0.2µM each GLUDG-L and CB2-H primers (Palumbi, 1996) and 0.5 units *Taq* DNA polymerase (Invitrogen, Carlsbad, California). Samples were run on 1.5% agarose gel stained with ethidium bromide to confirm amplification of the target fragment. Products within the expected size range were then sequenced to confirm the probe binding site was an exact match. To prepare for the sequencing reaction, excess reagents were eliminated using ExoSAP (New England Biolabs, Ipswich, Maine). Samples were sequenced using DYEnamic™ ET Dye Terminator chemistry (Amersham Biosciences Inc, Pittsburgh, Pennsylvania). Samples were run on a MegaBACE 1000 DNA Analysis system and analyzed with Sequence Analyser 3.0 (Amersham Biosciences Inc, Pittsburgh, Pennsylvania).

Samples which contained the desired sequences were quantified using fluorometer-based picogreen assay on the BMG FluoStar Galaxy 96-well plate system. The number of copies of the desired fragment was determined using molecular weights calculated for each control sample following the protocol outlined in Creating Standard

Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR (Applied Biosystems, copyright 2003). From the quantified product, serial dilutions containing 10^6,10^5, 10^4, 10^3, 10^2, 10 and 1 copy in a 5μl volume were made for all species. Two different samples for each species were quantified and diluted as technical replicates. In total six controls of amplified quantified mtDNA containing our target fragment were created using the above methodology.

Redesigning the assay for an additional mtDNA fragment for which flanking primers were not readily available, as well as the need to create a larger volume of high concentration control DNA, required cloning our target fragment for standard curve development (Pogozelski et al. 2003, Andreasson *et al.* 2006). Control samples for each species were amplified in a conventional PCR using the primers developed for the *Taq*man® assay. PCR product was visualized on a 1% low-melt agarose gel and stained with ethidium bromide. For products observed in the expected size range, bands were excised from the gel and placed in a 1.5 ml tube. Excised amplicons were heated to 65°C for 15 minutes in a water bath, then inserted into a bacterial vector following the protocol for gel-purified product in the TOPO cloning kit (Invitrogen, Carlsbad, California). Single white colonies were picked for screening, placed in 50μl $TE_{0.1}$ (Tris, 0.1mM EDTA) and boiled for 15 minutes to lyse the bacterial cells and denature cellular proteins. Each clone was amplified using M13 primers from the TOPO cloning kit (Invitrogen, Carlsbad, California) in a 100μl reaction containing 1x PCR buffer, 0.2mM of each dDNTP, 0.2μM each primer, 0.5 units *Taq* DNA polymerase (Invitrogen, Carlsbad, California) and 2μl of template and run on 1.5% agarose gel stained with ethidium bromide to confirm the insertion of DNA into the bacterial vector. Those

products within the expected size range were sequenced to confirm the exact nature of the inserted fragment. To prepare for the sequencing reaction excess reagents were eliminated using ExoSAP (New England Biolabs, Ipswich, Maine). Samples were sequenced using DYEnamic™ ET Dye Terminator chemistry (Amersham Biosciences Inc, Pittsburgh, Pennsylvania). Samples were run on a MegaBACE 1000 DNA Analysis system and analysed with Sequence Analyser 3.0.

Sequences were aligned in Bioedit 6.0 (Hall, 1999). Clones that contained the desired sequences were quantified using fluorometer-based picogreen assay on the BMG FluoStar Galaxy 96-well plate system. Each sample was quantified a minimum of three times to obtain an accurate value. In addition, quantification was confirmed by gel electrophoresis. The exact number of copies of the desired fragment was determined using molecular weight and calculated for each control sample following the protocol outlined in Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR (copyright 2003, Applied Biosystems). From the quantified product, dilutions of 10^6,10^5, 10^4, 10^3, 10^2, 10 and 1 copy in a 5μl volume were made for all species. Two different clones for each fragment were quantified and diluted for each species, as technical replicates. In total twelve controls were created using the above methodology.

For real-time PCR, a 20μl reaction was prepared containing 5μl of sample with a known quantity of mtDNA ranging from 10^6 copies to 1 copy, 1X PCR MasterMix and 0.3μM each of forward primer, and 0.3μM MGB probe (all Applied Biosystems, Foster City, California), and remaining volume of sterile deionized distilled water (Invitrogen, Carlsbad, California). DNA detection was performed using the Applied Biosystems 7900

sequence detection system. The reaction conditions for all assays were as follows; 10 minute activation at 95°C followed by 50 cycles of 15 seconds at 95°C and 1 minute at 55°C. Analysis of run data was performed using Sequence Detection Software version 2.1. For all reactions the threshold level was set at the mid-exponential position.

Analysis of Specificity

To determine the specificity of each assay, all species-specific reactions were performed on the other two elephant or mammoth species. In addition, all reactions including species-specific reactions were performed on mammals that are found in similar geographic locations, or those whose parts may be mistaken for those of elephant. This included dromedary camel, river hippopotamus, white rhinoceros and Indian rhinoceros, walrus, warthog and bovine. The Tagua palm produces a nut with a very white, hard cellulose kernel which can be worked in a similar fashion to ivory (Espinoza and Mann, 1991). A sample of this natural ivory substitute, or vegetable ivory, was also included to ensure that these types of samples would not result in false positives.

Reproducibility

To assess the accuracy and reproducibility of all reactions, a dilution series for all controls was run six times on three separate occasions. Means and variance of the threshold cycle (C_t) for each reaction as well as between all three runs were calculated to determine within and between run variability. Further statistical analysis was performed to assess the significance of run to run variation, and to determine if there were significant differences in C_t for the dilution series used to develop the standard curve. The

significance of between-run variance and copy number with respect to C_t and amount of DNA, as well as the interaction between these two variables were assessed using an analysis of co-variance (ANCOVA). The variable of DNA amount was further analyzed using a Tukey test, a multiple comparison procedure (Gotelli and Ellison, 2004) to test if the variance between amounts of DNA were significant in all pair wise comparisons. All statistical analysis was performed in XLSTAT 2008.6.03 (©Addinsoft 2008).

Evaluation of Sensitivity on Control DNA

A dilution series of control DNA extract was created for all three species and amplified using traditional PCR and all initial screening *Taq*man® assays in order to assess the sensitivity of the real-time assays as compared to traditional PCR. DNA from each of the three species was amplified in a 20µl volume using 1X PCR Gold Buffer, 1.5 mM $MgCl_2$, 2 mM of each dNTP (Invitrogen Life Technologies, Carlsbad, California), 0.2µM each primer, 1.25U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, California) and 5µl of template DNA. Samples amplified using traditional PCR were run on a 1.5% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

Despite international regulations the trade of ivory and elephant products remains a threat to the world's elephant populations (Stiles, 2004). The aim of this study was to design and validate a highly sensitive DNA based assay which may be used to accurately identify the presence of CITES-listed elephant species. Real-time PCR can be used to positively identify elephant specific DNA fragments in samples with limited quantity and poor quality DNA quickly and cost effectively.

*Taq*man®

Control development

In order to develop a standard curve, a source of mtDNA that could be quantified without the interference of nuclear DNA was required. In a human nuclear genome there is thought to be over 3 billion base pairs while there are only 16 569 in the mitochondrial genome (Butler and Levin, 1998). The higher ratio of nuclear DNA to mtDNA would bias quantification of total DNA obtained from the DNA extraction process to be primarily that of the nuclear contribution. In addition, single mitochondria may contain 2-10 copies of the mitochondrial genome, with a cell containing tens to thousands of mitochondria (Butler and Levin, 1998, Budowle *et al.* 2003 and Pakendorf and Stoneking, 2005). The variability in quantities of mtDNA per cell makes it difficult to estimate mtDNA contribution from quantification results of total DNA in a sample.

In this study we used two different methods to develop mtDNA controls to be used in quantifications of unknowns against a standard curve. Both methods were

comparable in their ability to produce accurate and reproducible curves. The initial methodology adapted from Alonso *et al.* 2004 required the existence or design of primers that flanked the target region. Using this method the flanking primers are used to amplify a portion of the mitochondrial genome which includes the target fragment. This amplified product can then be used as a quantifiable mtDNA control. As the control DNA extract originated from low template sample types such as nail clippings, blood, ivory and fossilized bone, the amplicon had to be a smaller portion of mtDNA; large fragments, such as the entire cytochrome *b* gene, were unlikely to amplify from degraded DNA extracts. For the second target fragment, there were no available primers to amplify a small portion of the cytochrome *b* region that included the target probe site.

The method applied by Alonso *et al.* (2004) also required a large quantity of control DNA extract for amplification. The large quantity of DNA extract was needed to perform large-scale amplifications for control development. Large volumes of PCR products to be used as mtDNA controls were required to perform replicate quantifications, and many replicate dilutions for validation of the controls and their subsequent use in evaluation of reproducibility and sensitivity. The use of the above methodology was not feasible given the requirement of flanking primers and low yields from the control samples.

As an alternative method of standard curve development, target fragments were cloned by inserting samplified product into plasmid vectors. Control DNA extract for all three elephant species were amplified using the primers designed for the *Taq*man® assays. This fragment was then cloned in a pCR 2.1-TOPO vector (Invitrogen, Carlsbad, California). Each clone was then amplified using M13 primers from the TOPO cloning

kit (Invitrogen, Carlsbad, California), and amplified product was quantified and diluted to create mtDNA controls.

This protocol eliminated the need for the design of flanking primers, as the flanking sequence is that of the plasmid for which primers are available. Other benefits to using plasmids as standard controls include harvesting large amounts of standards, long-term stability and ease of quantification (Pogozelski *et al.* 2003). Through this method of amplification we were able make enough PCR product for quantification, generation of standards and further optimization. The resulting standards remained in high quality throughout our testing as evidenced by the observed reproducibility and sensitivity.

The development of controls using a plasmid vector is more costly than the direct amplification method applied by Alsonso *et al.* (2004) as it requires the use of a PCR cloning kit. The up front cost of this method is worthwhile as the large quantity and high quality of standards produced means procedures do not have to be repeated. Using the direct amplification method, samples would have to be extracted more than once and the amplification repeated in order to obtain enough amplified product for replicate quantifications, dilutions and validation of the controls. Overall this increases the time required for control development using direct amplification, and also cost with respect to technician time. In addition, each new set of controls developed from amplified product would have to be validated. This testing increases the amount of technician time required, and may also introduce sources of error. Using a plasmid vector is a superior method for control development for *Taq*man® assays. This method allows for harvesting a large amount of standard from a small amount DNA extract, so isolation procedures, quantification and validation do not need to be repeated.

Analysis of specificity

For initial screening and all species-specific assays there was no cross-reactivity detected with DNA from dromedary camel, river hippopotamus, white rhinoceros, Indian rhinoceros, walrus, bovine, warthog or vegetable ivory. The lack of cross reactivity is most likely due to specificity of the forward primers. In the initial detection reaction for fragment 2 there was no cross-reactivity observed with hippopotamus DNA despite the absence of SNPs in the probe target site for this species. There are, however, seven SNPs in the forward priming site and this is likely the reason no cross reactivity was observed in the *Taq*man® assay. While the probe is able to hybridize, fluorescence is not observed as amplification does not occur due to the primer mismatch. This will cause a lack of 5' exonuclease activity of *Taq* DNA polymerase required to cleave the hybridized probe to cause fluorescence. It should be noted that the hippopotamus was the only species tested that did not show any SNPs at the probe target site. In addition there are many SNPs between hippopotamus and all other probe target regions. The observed lack of cross-reactivity in all other assays is likely the result of both the lack of amplification due to forward primer mismatch as well as the lack of probe hybridization.

The results for specificity testing for each assay on all elephant species are summarized in Table 4. We tested ten-fold dilutions of standards ranging from 10^6 copies to a single copy of elephant mtDNA per reaction. For both the fragment 1 and fragment 2 initial screening reactions we observed positive detection of fluorescence for all three elephant species in all template amounts tested. The initial screening assays showed the ability to distinguish our target taxa from others across a broad range of DNA template amounts.

The species-specific reactions for fragment 1 did not show specificity to the target species. High concentrations of DNA template from any of the three elephant species resulted in positive detection of fluorescence in all species-specific reactions, even when the template was not that of the target species. This was especially apparent in the case of African elephant and Woolly mammoth specific reactions. For fragment 1 there was only a single SNP which differentiates between African elephant sequences from those of the Woolly mammoth. The Woolly mammoth specific probe was designed to target this area. Despite targeting this SNP, African elephant DNA was detected when used as template in the Woolly mammoth reaction. One million copies of African elephant template resulted in the emission of fluorescence equal to 7.74×10^4 copies when quantified using the standard curve for Woolly mammoth. The probe designed for African elephant species identification did not incorporate the SNP that differentiates African elephant and Woolly mammoth. As expected, both African elephant and Woolly mammoth DNA were detected in the African elephant specific reactions. A template of 10^6 copies of Mammoth DNA was quantified as 9.6×10^5 copies in this reaction.

A single SNP was also not sufficient to distinguish Asian from African elephant. Asian elephant DNA was successfully detected in the African elephant reaction. The detection resulted in quantification of 10^6 copies as 2.1×10^3 copies when using the standard curve for African elephant. While the quantification is much lower than actual template amount, this result is not optimal. Detection of Asian elephant in the African elephant reaction, despite not being accurately quantified, would be a false positive result. New probes could not be redesigned for this fragment as there were no regions which showed higher interspecific variation.

Fragment 2 species-specific reactions showed no cross-reactivity observed with the other elephant species except when high amounts of either African or Asian elephant DNA were used as template in the Woolly mammoth-specific reaction. The resulting quantification from the addition of 10^6 copies of Asian elephant DNA to the Woolly mammoth reaction was 3.76 copies. Quantification results from the addition of 10^5, 10^4 and 10^3 copies were 0.88, 1.68 and 0.45 copies respectively. Similar results were obtained for African elephant template where the quantification from the addition of 10^6 copies of African elephant template was 0.38 copies, from 10^5 was 0.84 copies, from 10^4 was 0.62 copies and from 10^3 was 0.94 copies. Despite high probe specificity low levels of fluorescence were observed, which could be the result of non-specific binding of the probe. The level of detection in all cases was well below the lowest reliably quantifiable amount of template for our assays, as will be discussed in further detail below, and could be ignored in all cases.

Reactions involving sequences containing a single SNP are not ideal for species identification. When only one SNP was targeted with the *Taq*man® probe, we observed non-specific detection of non-target species. Similar results were reflected in a study by Itoi *et al.* (2005) where a single SNP was used to attempt to distinguish between two eel species, *Anguilla japonica* and *Anguilla anguilla.* The authors also found cross-reactivity between species and species identification was confirmed by assessing the differences in fluorescent intensities using a spectrophotometer and agarose gel electrophoresis. The post processing of samples in this manner is not ideal for forensic applications. Not only do post processing steps introduce sources of error, but forensic evidence based on judgment calls and not definitive results are difficult to defend in court.

Species identification assays have been successfully designed when the probe does not exactly match the target species. The probe designed by Lopez- Andreo *et al.* (2005) to detect cow, sheep and pig in complex mixtures had a SNP in the pig DNA target. Reactions were made specific in this study through the use of species-specific primers. The authors were able to detect pig in mixed samples when pig DNA was 2-5% of the total sample. In our study detection can occur despite a mismatch in the probe site, and reactions can be made specific using primers. We increased the number of SNPs in our species-specific probes to reduce cross reactivity, in addition to utilizing elephant specific primers.

Through targeting additional SNPs in the fragment 2 probes we essentially eliminated any cross reactivity. Most other studies using SNP methodology for identification of species incorporate more than one SNP, including a protocol intended for human forensic casework, where 3 SNPs were used to distinguish between very distantly related species including human, domestic dog and cat (Nakaki *et al.* 2007). We have incorporated three or more SNPs between elephant species in each of our probe target regions and have demonstrated a lack of cross reactivity between all elephant species as well as non target species. Based on our results and the supporting literature, fragment one was no longer considered useful for species identification. The probe designed for initial screening in fragment 1 showed adequate specificity and was further investigated for reproducibility. Initial screening with the primers and probe designed for fragment 1 could be used to strengthen results from screening with fragment two.

Reproducibility

All elephantid detection and species-specific assays showed consistent and reproducible results in control samples with 10^6 to 10^2 copies of mtDNA. An example of typical control amplification plot is shown in Figure 7. This plot shows the exponential increase in fluorescence over time for a ten fold dilution series of our Woolly mammoth control samples from 10^6 to a single copy. The threshold cycle is plotted in the middle of the exponential phase of the reaction. Samples with higher amounts of template show an exponential increase in fluorescence and cross the threshold line at earlier PCR cycles than those with low template amounts. A typical control standard plot is shown in Figure 8. The standard plot is generated by plotting the amount of template DNA in a sample against the threshold cycle observed for that sample. The linear relationship between these two variables allows for the quantification of unknowns through plotting C_t to determine the starting template amount. The success rate for all replicates as well as means and variance of C_t within and between all three runs are summarized in Table 5. Samples with 10 copies amplified 99 percent of the time and those with only a single copy amplified 90 percent of the time. There is a higher variance in C_t observed between runs than that observed within, and in most cases the variance in C_t is higher for samples with less than 10^2 copies of starting template. Box and whisker plots showing means, 1st and 3rd quartiles, 95% confidence limits and maximum and minimum values for C_t for initial screening reactions and species detection of Asian elephant are shown in figures 9, 10 and 11 respectively. Box and whisker plots for initial screening and species detection for all other species can be found in Appendix I. These plots highlight the increased variance in C_t for lower template amounts and show the lack of differentiation in C_t for

those samples with templates of 10 and 1 copy. Figure 9 shows a high degree of overlap in C_t ranges for samples with 10 copies and 1 copy, even when variation is low. Figure 10 highlights the high degree of variation observed in samples with less than 10^2 copies of template DNA. For both of these assays the resulting standard curve would be poor due to a loss of linearity.

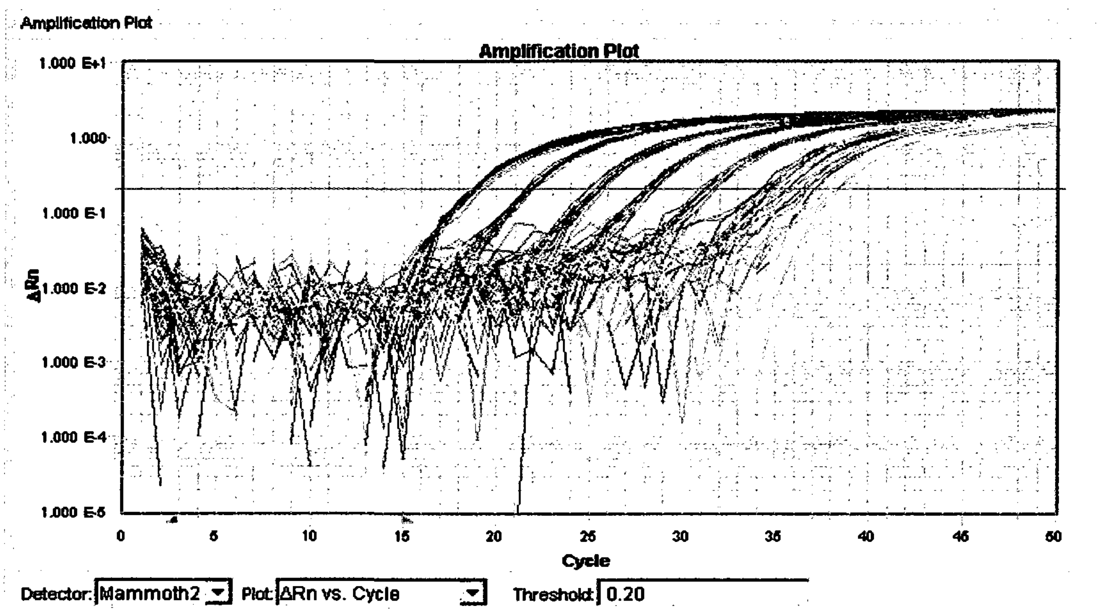

Figure 7. Amplification plot of dilution series of Woolly mammoth DNA amplified using species-specific reaction. The threshold cycle is indicated by the red line. Samples with 10^6 copies of template cross the threshold first around cycle 19, followed by 10^5, 10^4, 10^3, 10^2, 10 and 1 copy.

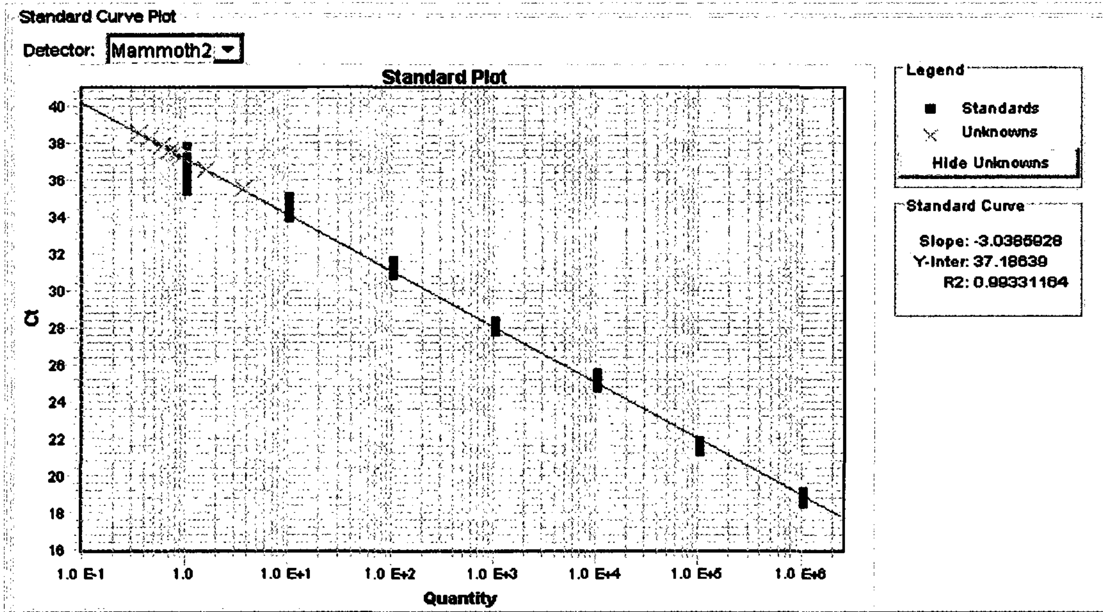

Figure 8. Standard plot of threshold cycle and DNA template amount for a 6-fold dilution series ranging from 1 million to 1 copy of Woolly mammoth mitochondrial DNA, amplified in species-specific reaction. The resulting standard curve can be used for quantification of Woolly mammoth DNA in unknown samples.

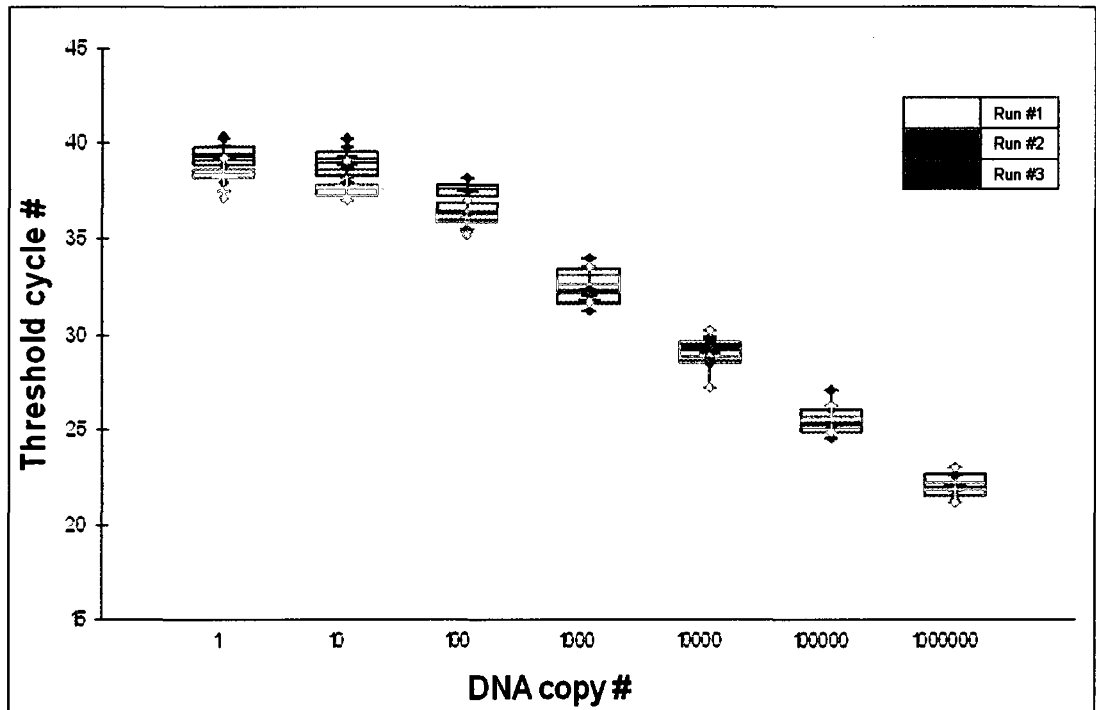

Figure 9. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

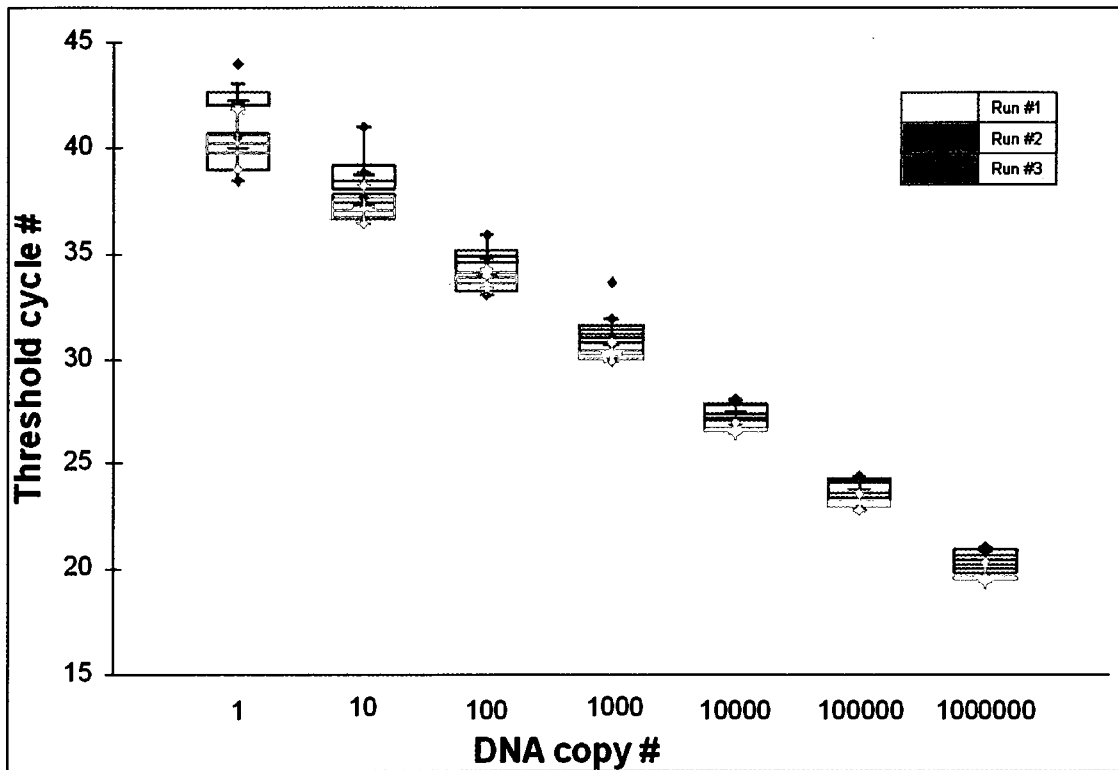

Figure 10. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

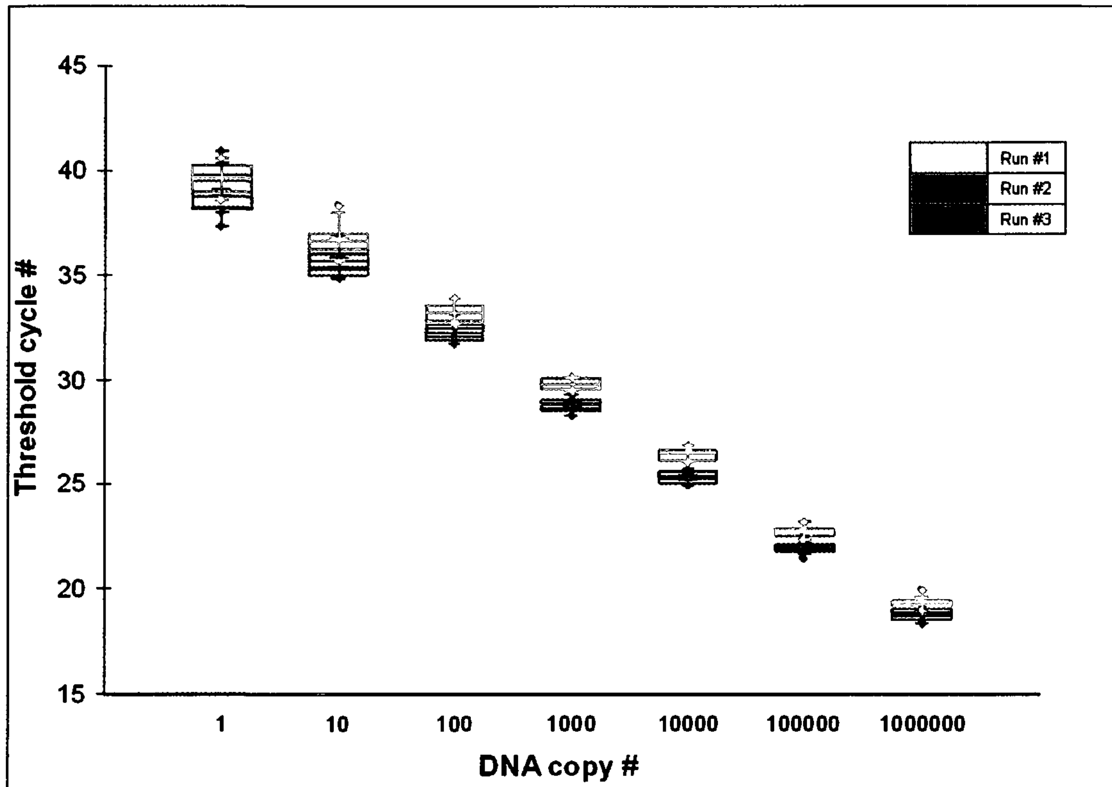

Figure 11. Box and whisker plots for three real-time PCR runs of Asian elephant species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Asian elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

The results of statistical testing with ANCOVAS are summarized in Table 6. The variation in C_t observed between replicate runs of the same assay is not significant, while template amount is a significant variable with respect to C_t. An exception is the between run variance in C_t for fragment 1 initial screening assay with Woolly mammoth template, where the variance in C_t was significantly different ($p=0.01$) between runs. This difference in variance was the result of a single run with lower C_t values than for the other two replicates and is most likely the result of human error. Further analysis of the ANOCOVA results with a Tukey test showed that there was a significant difference in all

pairwise comparisons of template amount. The lone exception to this was the pairwise comparison of 1 to 10 copies in the fragment 1 initial screening assay with Asian elephant template. As discussed above, variance increased with template amounts below 10^2 copies and for samples with 10 and 1 copy, C_t values fell within a similar range for this assay.

An increase in the variability in detecting low template amounts has also been observed in other studies. Lopez-Andreo *et al.* (2005) observed a loss of linearity in their standard curve for samples showing results above 40 cycles. For quantification of human mtDNA by Von Wurmb-Schwark *et al.* in 2002, an increase in standard deviation occurred in low copy number samples. For those samples with only 10 copies the standard deviation increased to 32.1% as compared to 11.8% for 10^2 copies. The authors determined the lowest reliably quantifiable number of input copies was 10 molecules of mtDNA, despite the higher standard deviation.

In addition to an increase in variability, samples with 10 copies or fewer did not amplify in every test. These results are likely due to stochastic variation in PCR (Von Wurmb-Schwark *et al.* 2002) or variability associated with dilution and pipetting of low quantities of DNA. Despite a number of failed reactions our success rate is comparable to others. Our success rate for single copies of 90% across assays is greater than that reported by Wetton *et al.* (2002), who had a 63% success rate with single molecules. Analysis of human mtDNA in bone samples could detect a single copy although the incidence of failures increased when there were less than 60 copies in the original sample (Alonso *et al.* 2003). This success rate observed for samples with a single copy is an important factor in determining the quality of each reaction. Controls with a single copy

template are expected to show fluorescence a good portion of the time. If this is not the case it indicates a problem with the reaction as there is a decrease in sensitivity. A dilution series of controls including those with a single copy should be run on every plate to ensure the quality and sensitivity of the *Taq*man® assay.

We have determined the lowest reliably quantifiable amount of template in our reactions is 10^2 copies. Below 10^2 copies we observed an increase in sample variability and less accurate quantification, due to non-linearity in our standard curve. A PCR of more than 40 cycles can increase the amount and complexity of non-specific background products (Innis *et al.* 1990). For real-time PCR this increase in non specific product may result in the detection of fluorescence above what is expected in a sample without non-specific products, causing a non linear standard curve. In all assays designed in this study, samples with $\geq 10^2$ copies of template showed detectable fluorescence before 40 cycles had been completed. In addition we observed 100 % success rate in all samples with $\geq 10^2$ copies of template. As our assay is intended for forensic purposes, a cut off of 10^2 copies is conservative and easily defendable in a court setting. Samples above a 10^2-copy threshold will show 100% success and high reproducibility and no cross-reactivity.

Table 7 shows the slopes, reaction efficiencies and R^2 values for all runs when only template amounts of $>10^2$ are used in calculations. Reaction efficiency was calculated using the slope of the standard curve where $E_x = 10^{(-1/slope)}-1$. In all cases R^2 values were high ranging from 0.98 in a single fragment 1 initial screening reaction to 0.99 in all others. The reaction efficiency observed in some runs of the fragment 1 initial screening reactions were low. The values for fragment 1 ranged from 76% to 101% as compared to 86% to 103% in the fragment 2 initial screening reactions and 96% to 113%

in the species-specific reactions. The reaction efficiencies for fragment 1 initial screening are concerning when one considers that reactions with poor efficiency will have poor sensitivity, particularly in low copy number samples (Peters et al. 2004).

In addition to reduced reaction efficiency in the initial screening reaction for fragment 1, there was an increase in observed variance in C_t compared to that for fragment 2. In particular, the Woolly mammoth reactions using fragment 1 had a variance greater than 1 cycle even with more than 10^2 copies of starting template, while this variance was only 0.1-0.2 cycles for fragment 2. This assay also showed the only R^2 value below 0.99 with a value of 0.98. In contrast, fragment 2 showed more consistent results with R^2 values of 0.99 and a variance in C_t of less than one PCR cycle for all samples with $>10^2$ copies of starting template. The same level of consistency was also observed for all species-specific reactions targeting the same region. As a result, the initial screening for elephant in our protocol was completed using the universal primers and probes designed for fragment 2.

The proposed workflow for elephant species detection and quantification is shown in figure 12. The protocol involves an initial screening reaction using a universal probe designed to target a region of variability between elephants and other closely related mammals. This initial screening will determine whether a sample contains elephant DNA from any of our three species of interest, and the quantity of elephant DNA in the sample. If this test shows the presence of elephant DNA at a concentration higher than 10^2 copies, species-specific reactions will then be performed to determine the species of origin.

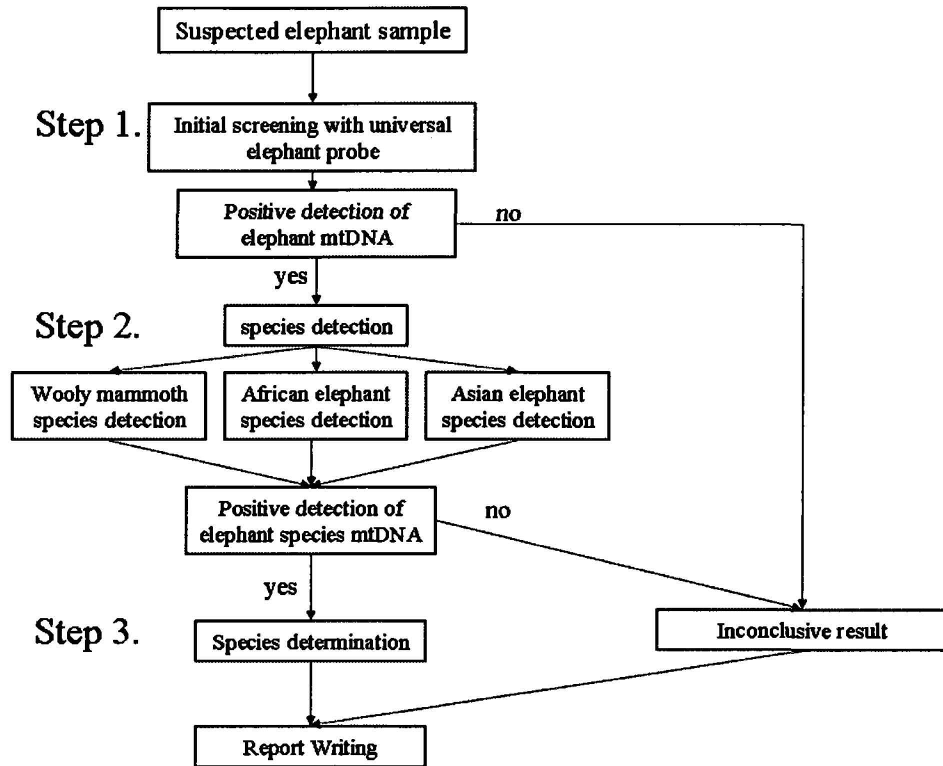

Figure 12. Decision making tree and proposed workflow for elephant species determination using real-time PCR assays designed in this study.

Our protocol also requires that two control samples for each dilution ranging from 1 million to a single copy, and controls for each probe target should be run on every plate. For initial screening this would involve running controls for all three species. Despite demonstrating reproducibility between runs, there were slight differences in C_t values between runs. Figure 11, which shows box and whisker plots for three runs of the Asian elephant species-specific assay, highlights the need for controls in each run. The C_t values for run one were slightly higher than for runs two and three. The difference observed, while not significant in statistical testing, is significant with respect to accurate quantification. If samples are quantified with a standard curve generated from a different run the quantification will not be accurate. For example in figure 10, an unknown sample

from run 1 would show less DNA when quantified with standard curves from runs 2 or 3. In order to obtain the most accurate quantification of a sample, the amount of DNA should be determined using a standard curve from controls from the same run. A protocol that includes running controls on every run will ensure that samples are quantified as accurately as possible despite slight run-to-run variation. Duplicates of controls will allow for the estimation of the within run variance to show confidence levels for quantification results.

Evaluation of sensitivity on control DNA

The results of traditional PCR reactions using fragment 2 primers and African elephant DNA are shown in figure 13 (see appendix for other species). Gel images for amplifications using fragment 1 primers are shown in Appendix II. To evaluate sensitivity we considered traditionally amplified samples that exhibited at least 15 nanograms of amplified product to be positively detected on an agarose gel. This is the minimum amount of amplified product required for DNA sequencing (DYEnamic™ ET Dye Terminator chemistry manual , Amersham Biosciences Inc, Pittsburgh, Pennsylvania). Results of *Taq*man® amplification for the same samples are summarized in Table 8. Both methods showed a similar range of detection for all three species. The lowest dilution exhibiting detection for our African elephant control DNA from a blood sample was 1/125th dilution of stock, Woolly mammoth DNA from a fossilized sample was > 1/5th dilution of stock and for Asian elephant DNA from a toenail sample was 1/3125th dilution of stock.

Figure 13. Dilution series of African elephant stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase. Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel.

We observed no evidence of increased sensitivity in our *Taq*man® reaction as compared to traditional PCR with hot start *Taq*. Cases where an increase in sensitivity was observed were those where the original methods used are less sensitive than traditional PCR methodologies: Fox *et al.* 2005, observed an increase in detection sensitivity as compared to iso-electric focusing (IEF) method for egg species identification; Alonso *et al.* 2004 showed higher sensitivity as compared to slot-blot hybridization where 30% of bone samples that had given a negative result using the slot-blot method had positive results with real-time PCR; Andreasson *et al.* 2002 also compared real-time PCR to slot blot and found the sensitivity of real-time PCR to be higher. This study also compared the sensitivity of real-time PCR to the AluQuant® Human DNA Quantitation System (Promega, Madison Wisonsin) and the authors found the AluQuant® had a higher sensitivity; however this system requires more time due to a limit of 16 samples per quantification run.

While there was a similar sensitivity in traditional PCR with hot start *Taq*, utilizing a 1.5% agarose gel for visualization, we did not sequence these products to

confirm their utility for species identification. Sequencing results are not always of high quality despite evidence of good quality template. It is possible that while we observed a band on a gel we would not be able to accurately identify species from the amplified product. This is not the case with real-time PCR where the observation of amplification is in itself positive evidence of the presence of an elephant species.

Benefits of real-time PCR over DNA sequencing

Species identification of traditionally amplified product is often accomplished using DNA sequencing (Bataille *et al.* 1999, Parson *et al.* 2000, Branicki *et al.* 2003 and Bravi *et al.* 2004). Unlike real-time PCR where detection shows positive identification, there is no guarantee of obtaining a good quality sequence from samples which show amplification success with traditional PCR. In our study we amplified DNA from a Woolly mammoth tusk obtained from the Canadian Wildlife Service. The amplified product was easily visualized on an agarose gel and the resulting sequence had a 95% quality score assigned by the sequence analysis software. Figure 14 shows the base called sequence from Sequence Analyzer v. 3.0 software. Despite the high quality score there were a number of areas where the sequence quality is questionable. There are many causes for poor quality sequencing results, including adding too much or too little DNA into the sequencing reaction, sample contamination or mixtures of DNA in the sample and heteroplasmy (Melton and Nelson, 2001).

DNA sequencing requires a set amount of DNA to be added to the sequencing reaction. For the sequencing reactions performed in this study, 10 nanograms of amplified product were recommended for every 100 base pairs of desired sequence

(DYEnamic™ ET Dye Terminator chemistry manual, Amersham Biosciences Inc, Pittsburgh, Pennsylvania). Other sequencing chemistries also have stringent template requirements. For example, Applied Biosystem's Big Dye version 3.1 chemistry requires 2.5 nanograms for every 100 base pairs of desired sequence (Big Dye terminator version 3.1 cycle sequencing kit protocol, Amerhsam Biosciences, Grove City, California). When less DNA is added to the sequencing reaction, sequences may be of poor quality due to low fluorescence in the sequencing products. This will result in poor or the complete lack of base calling by sequence analysis software. Adding too much DNA into a sequencing reaction will cause poor amplification of the entire fragment as reagents are consumed very quickly in the first few cycles of amplifications. The resulting sequencing product will exhibit strong fluorescence in the first few bases with declining fluorescence throughout the rest of the sequence (MegaMANUAL, Amersham Biosciences Inc, Pittsburgh, Pennsylvania). In both cases base calling by the sequence analysis software will be negatively affected. By contrast we have shown that real-time PCR shows accurate and reproducible results over a wide range of template amounts.

Often sequences can have ambiguous bases and artefacts that can make interpretation difficult. This ambiguity may be difficult to explain or support in a court setting. This is because ambiguous bases could indicate contamination. When DNA from more than one source is amplified, the resulting sequence will most likely have numerous sites where more than one base is present. If DNA from a single source exhibits the same type of ambiguity, it calls into question the techniques and protocols used in generating those results.

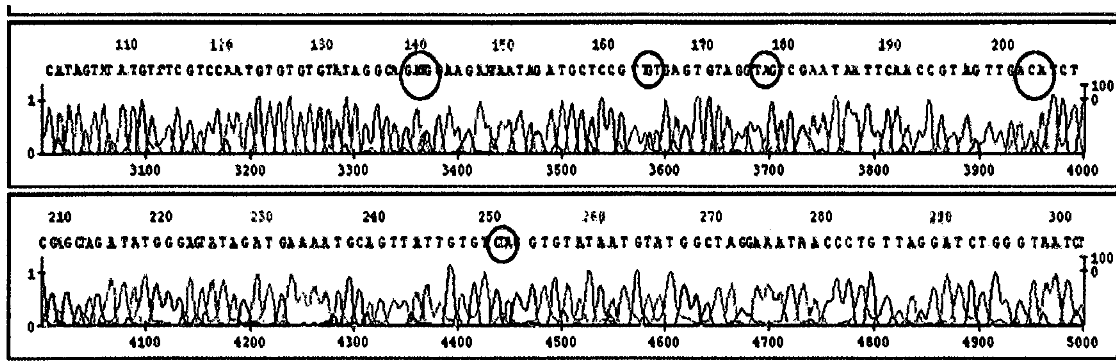

Figure 14. A portion of a Cytochrome *b* sequence from amplification and sequencing DNA extracted from a Woolly mammoth ivory sample. Red circles indicate regions of ambiguity in base calling in the interpreted output from Sequence Analyser 3.0 (Amersham Biosciences Inc, Pittsburgh, Pennsylvania)

Another cause of ambiguous base calling is heteroplasmy, which occurs when more than one sequence of mtDNA exists in the same individual (Melton and Nelson, 2001 and Budowle *et al.* 2003). Heteroplasmy is common and well documented in the hypervariable regions of the human mtDNA genome. In a survey of two years of human forensic mtDNA analysis heteroplasmy was observed in 5.7% of samples and in most cases the heteroplasmy was observed at sites in which it had been previously documented (Melton and Nelson, 2001). Heteroplasmy is not as well documented in other species, and would have to be confirmed by replicating both the original PCR and sequencing reactions.

Heteroplasmy will have minimal or no effect on real-time PCR results. If the heteroplasmy occurs in a portion of the DNA fragment that is not targeted by the *Taq*man® probe, heteroplasmy will have no effect on the results of the assay. If heteroplasmy or mutations exist in the region targeted by the probe the resulting quantification would be lower than the actual DNA amount although identification will

still be possible. In extreme cases this may cause a sample to fall under our minimum quantification threshold and species of origin will not be determined. In a legal context this means our assay is biased towards exonerating a defendant if issues related to heteroplasmy are encountered.

When mtDNA sequencing evidence is used in court all of the above issues may be raised as possible reasons to exclude it. For example, the first Canadian case where mtDNA was admitted as evidence in a human case was in 1999 in the case of R. vs. Murrin. In this case the defendant argued that with mtDNA the potential for contamination and erroneous results was so high that the results were inherently unreliable. Secondly, he argued that heteroplasmy provides further uncertainty. The combination of these uncertainties, said the accused, demonstrates that mtDNA analysis was not yet ready for the courtroom. Despite these arguments by the defence, mtDNA analysis was allowed as evidence. In this, and many other cases where mtDNA evidence has been admitted in court in the United States, samples were processed by different laboratories and sequences analyzed by at least two individuals in order to confirm sequence results (R. vs. Murrin, 1999). This independent replication is the best way to eliminate contamination and biased interpretation as reasons to throw out mtDNA evidence from a case.

The principles of real-time PCR eliminate many of the above variables that make DNA sequencing problematic. First we have demonstrated that real-time PCR shows accurate and reproducible results over a 5 log scale of template amounts, where sequencingrequires a very specific amount of DNA template. Secondly, the difficulties in base calling associated with ambiguous bases or heteroplasmy are eliminated. A

sample is either shown to contain DNA from the target species, or not. If heteroplasmy or mutations exist in our probe target region our assay is biased toward exclusion and would show less quantifiable DNA or no result at all. There is no interpretation of questionable results as is required with DNA sequencing.

The results obtained from real-time PCR are explicit. Using a strict cut off for detection of 10^2 copies, and providing a strongly supported linear curve ($R^2 \geq 0.99$), samples are either positively identified as containing elephant or not. Following initial detection samples are identified as one of three species. Quantification results from the elephant detection assay and the species identification should be comparable, providing an additional line of evidence for positive species identification through demonstrating reproducibility in two independent tests. An assay with a discrete presence or absence result is more desirable as evidence in court proceedings as it leaves little room for the argument of biased interpretation.

Another benefit to real-time PCR is the cost effectiveness. Table 9 shows the breakdown of costs associated with the two methodologies. The values reflect the cost of processing 48 case samples in a 96 well plate format. For sequencing this includes processing 48 case samples as well as a controls for each species and two no template controls or negatives one from extraction and the other from PCR. Sequencing costs also include 4 sequencing reaction controls which should be run on every plate. Cost analysis assumes that a high quality sequence is obtained the first time a sample is run and does not include sequencing in the opposite direction or confirming a sequence through replication. Real-time PCR costs include 4 runs for each sample, 1 initial screening reaction and three species determination reactions and the cost of running positive

controls for each species and 2 negative controls on each plate, one from extraction and one for real-time PCR. Using the protocols designed in this experiment, positive identification of species can be obtained directly from the real-time PCR reactions. Using traditional PCR, extracted samples would be amplified, run out on a gel to confirm amplification, cleaned and sequenced, then analyzed. The sequencing process requires more reagents, takes more time and requires more hands on technician time.

There is also higher cost associated with analysis of sequencing data. Sequencing analysis involves the manual inspection and interpretation of base-called data, sequence alignment and comparison against database samples, as well as phylogenetic and statistical analysis before the report is written. With real-time PCR samples are automatically analyzed upon completion of the reaction. The analyst would confirm that control samples amplified as expected and the R2 value is $\geq$0.99, and a report would be written.

Reducing the number of processing steps in an assay is beneficial with respect to forensic case work. First, results can be obtained much faster when only a single step is required, this would enable a laboratory to process more samples, as well as provide forensic evidence in a relatively short period of time. The overall reduction in cost would also allow investigators to process more samples. This could include more samples related to a single case to increase the amount of evidence, or samples from additional cases that otherwise may not be analyzed due to budget. Finally, less processing greatly reduces the chances of contamination or error. This provides little room for the argument in court that a sample was mishandled or contaminated during DNA analysis.

CONCLUSION

The real-time PCR technique enables rapid and cost-effective identification for both CITES listed elephant species and the extinct Woolly mammoth. Both elephant detection and species determination assays showed consistent and reproducible results in samples with a wide range of template amounts, and as low as 10^2 copies. Those samples with fewer than 10^2 copies were detected the majority of the time, but had a higher variance in C_t and some failures in samples with low template amounts. Where possible, as a safeguard, larger quantities of DNA should be used when analyzing forensic materials (Andreasson *et al.* 2002).

Another important consideration when designing a *Taq*man® assay, is the sequence differentiation in the probe target region. It is important to maximize the number of SNPs between the target species and closely related species in the *Taq*man® probe. When only a single SNP is targeted the reaction may not demonstrate specificity to a particular species. A probe targeting three or more SNPs showed specificity for all three species in this study.

The results of this study clearly demonstrate the utility of *Taq*man® real-time PCR technology in wildlife forensics for species identification. Unlike traditional methods of elephant identification from ivory, our assay can potentially be used to identify species in all elephant tissues including leather and hair. Positive identification can be obtained from a small sample with very little processing and analysis time. The reduced processing time increases the power of results by reducing the chances for contamination and error, as well as significantly reducing the costs associated with DNA

analysis. Results should be admissible in court as strong evidence without the ambiguity associated with sequencing analysis.

Through Environment Canada enforcement, our country is making an impact in global conservation, but this impact is limited by the number of products that can be accurately identified. The development of the above species identification method will increase the potential to protect endangered species worldwide. Because this real-time PCR assay is less time consuming and expensive compared to DNA sequencing it may increase the number of cases that may be processed by a laboratory at little or no additional cost to the requestor. Increasing the number of cases that can be processed, may impact the number of cases tried, as a much stronger cases will be made in court with the support of accurate species identifications.

Future studies into this technology may involve identification of other endangered species. The specificity and the ability to quantify DNA in this type of assay would make it particularly useful in identification of species used in Traditional Asian medicines. In these types of products there may be complex mixtures of many species including plants and animals. As more protocols are developed there will a global impact on illegal harvesting as it will become increasingly difficult to illegally trade CITES listed species.

Table 1. Total number of single nucleotide polymorphisms (SNPs) between *Taq*man® probe target species and other non target elephant species

	Number of SNP's between non-target species		
Probe	African	Asian	Mammoth
African1	0	1	0
Asian1	2	0	2
Mammoth1	1	3	0
African2	0	3	2
Asian2	4	0	6
Mammoth2	3	6	0

Table 2. Sequences and fluorescent labels for all primers and probes used in elephant species identification assays.

Name	Primer/ Probe	Sequence	5' Label
Elephant1F	Primer	CACACACATTGGACGAAACATCT	none
Elephant1R	Primer	TGCCCCTCAGAATGATATTTGT	none
Elephant1	Probe	ACCATAGCCACCGCCT	TET
African1	Probe	CTATGGGTCCTACCTATA	6FAM
Asian1	Probe	TGGATCCTACCTATACTCAG	VIC
Mammoth1	Probe	AAATACCGGCATTATACTAC	TET
Elephant2F	Primer	CCATCCTACGATCTGTACCAAACAAAC	none
Elephant2R	Primer	CTTCGGTGCTTGGATGTATG	none
Mammoth2R	Primer	CATACTTCGGTGTTTAGATGTA	none
Elephant2	Probe	AGGCGTCCTAGCCCT	TET
African2	Probe	CCTAGGATTAATACCACTTCT	6FAM
Asian2	Probe	TGATTTTAGGATTAATACCATTTCT	VIC
Mammoth2	Probe	CTAATCCTAGGAATTATACCACTTCTA	TET

Table 3. Primer and probe combinations for elephant species identification assays designed for two different portions of the mitochondrial cytochrome *b* region.

Assay	Purpose	Forward Primer	Reverse Primer	Probe	Amplicon Length (bp)
Elephant1	Initial Screening	Elephant1F	Elephant1R	Elephant1	145
African Elephant1	Species Identification	Elephant1F	Elephant1R	African1	145
Asian Elephant1	Species Identification	Elephant1F	Elephant1R	Asian1	145
Wooly Mammoth1	Species Identification	Elephant1F	Elephant1R	Mammoth1	145
Elephant2	Initial Screening	Elephant2F	Elephant2R, or Mammoth2R	Elephant2	103 elephant, 106 mammoth
African Elephant2	Species Identification	Elephant2F	Elephant2R	African2	103
Asian Elephant2	Species Identification	Elephant2F	Elephant2R	Asian	103
Wooly Mammoth2	Species Identification	Elephant2F	Mammoth2R	Mammoth	106

Table 4. Cross reactivity observed for elephant detection and species identification assays between other non-target elephant species. Table values are amount of template DNA (copies) detected using standard curve quantification for the target species.

Template Source	Elephant Universal Fragment 1	Elephant Universal Fragment 2	Asian elephant Fragment 1	African elephant Fragment 1	Woolly mammoth Fragment 1	Asian elephant Fragment 2	African elephant Fragment 2	Woolly mammoth Fragment 2
Asian elephant 10^6	1000000.00	1000000.00	1000000.00	2146.25	0.00	1000000.00	0.00	3.76
Asian elephant 10^5	100000.00	100000.00	100000.00	149.38	0.00	100000.00	0.00	0.88
Asian elephant 10^4	10000.00	10000.00	10000.00	11.10	0.00	10000.00	0.00	1.68
Asian elephant 10^3	1000.00	1000.00	1000.00	0.00	0.00	1000.00	0.00	0.45
African elephant 10^6	1000000.00	1000000.00	5.24	1000000.00	77444.27	0.00	1000000.00	0.38
African elephant 10^5	100000.00	100000.00	0.01	100000.00	8988.28	0.00	100000.00	0.84
African elephant 10^4	10000.00	10000.00	0.00	10000.00	1424.94	0.00	10000.00	0.62
African elephant 10^3	1000.00	1000.00	0.00	1000.00	179.86	0.00	1000.00	0.94
Woolly mammoth 10^6	1000000.00	1000000.00	781.29	958787.50	1000000.00	0.00	0.00	1000000.00
Woolly mammoth 10^5	100000.00	100000.00	26.54	118896.38	100000.00	0.00	0.00	100000.00
Woolly mammoth 10^4	10000.00	10000.00	1.13	1032.58	10000.00	0.00	0.00	10000.00
Woolly mammoth 10^3	1000.00	1000.00	0.00	134.87	1000.00	0.00	0.00	1000.00

Table 5. The success rate for all replicates as well as means and variance within and between all three runs for elephant detection assays and three elephantid species.

			Run 1		Run 2		Run 3		Total		
Reaction	Species	Quantity	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	% Success
Elephant Universal Fragment 1	Asian Elephant	1000000	21.86	0.32	21.98	0.11	22.09	0.39	21.98	0.27	100
		100000	25.44	0.18	25.48	0.08	25.39	0.57	25.44	0.26	100
		10000	28.94	0.72	29.37	0.08	29.06	0.23	29.12	0.36	100
		1000	32.64	0.30	31.80	0.15	33.03	0.33	32.49	0.52	100
		100	36.07	0.24	36.47	0.45	37.47	0.34	36.67	0.68	100
		10	37.64	0.33	39.27	0.13	38.90	0.64	38.60	0.84	100
		1	38.32	0.32	39.47	0.22	39.22	0.41	39.01	0.55	100
	African Elephant	1000000	19.54	0.27	19.37	0.19	20.40	0.19	19.77	0.41	100
		100000	23.22	0.47	22.84	0.19	23.98	0.22	23.35	0.51	100
		10000	26.43	0.07	26.61	0.13	27.14	0.15	26.73	0.20	100
		1000	30.23	0.16	29.85	0.28	31.02	0.15	30.37	0.43	100
		100	33.68	0.22	33.87	0.09	34.57	0.45	34.04	0.39	100
		10	36.78	0.26	36.62	0.24	37.48	0.26	36.96	0.38	100
		1	38.96	0.40	38.12	1.49	38.81	0.61	38.63	0.92	100
	Wooly Mammoth	1000000	20.07	0.68	21.44	0.46	21.91	0.49	21.14	1.14	100
		100000	23.86	0.94	25.08	0.44	25.38	0.80	24.77	1.13	100
		10000	27.51	0.94	28.92	0.26	29.23	0.67	28.55	1.17	100
		1000	31.15	0.68	33.09	0.42	33.29	0.23	32.51	1.37	100
		100	34.89	0.31	37.26	0.35	37.83	0.34	36.66	1.98	100
		10	37.83	0.20	40.91	0.58	41.29	0.68	40.01	2.93	100
		1	39.59	0.36	42.78	0.95	43.18	0.66	41.85	3.27	100

Table 5 continued. The success rate for all replicates as well as means and variance within and between all three runs for elephant detection assays and three elephantid species.

			Run 1		Run 2		Run 3		Total		
Reaction	Species	Quantity	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	% Success
Elephant Universal Fragment 2	Asian Elephant	1000000	19.62	0.06	20.23	0.26	20.44	0.26	20.10	0.30	100
		100000	23.08	0.05	23.65	0.42	23.85	0.32	23.53	0.36	100
		10000	26.66	0.02	27.23	0.48	27.44	0.17	27.11	0.32	100
		1000	30.23	0.09	30.76	0.70	31.43	0.65	30.81	0.70	100
		100	33.81	0.10	33.90	0.53	34.89	0.22	34.20	0.52	100
		10	37.14	0.33	37.36	0.54	38.79	1.11	37.76	1.18	100
		1	40.24	0.74	40.01	1.46	42.27	1.14	40.71	1.95	72
	African Elephant	1000000	19.68	0.05	19.85	0.06	20.17	0.42	19.90	0.21	100
		100000	22.84	0.18	22.93	0.03	23.75	0.60	23.18	0.43	100
		10000	26.34	0.32	26.46	0.04	27.03	0.31	26.61	0.31	100
		1000	29.46	0.26	29.92	0.05	30.91	0.14	30.10	0.51	100
		100	32.78	0.38	33.20	0.09	34.61	0.07	33.53	0.80	100
		10	35.77	0.15	36.16	0.17	39.24	7.47	36.68	3.24	92
		1	39.38	1.92	39.40	0.58	40.17	1.16	39.39	1.16	64
	Wooly Mammoth	1000000	20.44	0.10	20.70	0.06	20.12	0.06	20.42	0.13	100
		100000	23.81	0.07	23.96	0.13	23.61	0.11	23.80	0.12	100
		10000	27.60	0.03	27.66	0.45	27.24	0.04	27.50	0.20	100
		1000	31.08	0.02	31.53	0.22	30.97	0.03	31.19	0.15	100
		100	34.69	0.04	35.47	0.12	34.75	0.05	34.97	0.19	100
		10	38.08	0.15	39.40	3.15	38.56	0.30	38.68	1.44	100
		1	40.13	0.86	42.89	5.38	41.94	1.71	41.62	3.76	97

Table 5 continued. The success rate for all replicates as well as means and variance within and between runs all three species identification reactions.

			Run 1		Run 2		Run 3		Total		
Reaction	Species	Quantity	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	Mean (C_t)	Variance (n-1)	% Success
Asian Elephant	Asian Elephant	1000000	19.34	0.08	18.68	0.05	18.84	0.05	18.95	0.14	100
		100000	22.69	0.06	21.83	0.04	22.07	0.03	22.20	0.17	100
		10000	26.41	0.10	25.21	0.04	25.43	0.03	25.68	0.33	100
		1000	29.80	0.08	28.67	0.04	28.99	0.02	29.15	0.27	100
		100	33.19	0.16	32.07	0.05	32.47	0.13	32.58	0.33	100
		10	36.69	0.63	35.29	0.14	35.83	0.34	35.94	0.68	100
		1	39.59	0.61	38.85	0.91	39.03	0.97	39.09	0.89	86
African Elephant	African Elephant	1000000	18.02	0.06	18.46	0.08	18.18	0.05	18.22	0.09	100
		100000	21.23	0.02	20.81	0.26	21.55	0.08	21.20	0.21	100
		10000	25.01	0.04	24.25	0.27	25.55	0.10	24.94	0.43	100
		1000	28.16	0.04	27.90	0.21	28.98	0.01	28.35	0.30	100
		100	31.55	0.02	31.23	0.16	32.22	0.05	31.67	0.25	100
		10	34.48	0.17	34.20	0.42	35.58	0.26	34.75	0.63	100
		1	36.07	0.43	37.71	0.96	38.51	0.58	37.37	1.66	94
Wooly Mammoth	Wooly Mammoth	1000000	18.77	0.04	18.40	0.05	18.78	0.05	18.65	0.07	100
		100000	21.72	0.03	21.85	0.04	22.29	0.06	21.95	0.10	100
		10000	25.33	0.06	25.23	0.04	26.36	0.04	25.64	0.31	100
		1000	28.15	0.04	28.88	0.03	29.98	0.08	29.00	0.63	100
		100	31.40	0.08	32.20	0.04	33.20	0.14	32.27	0.64	100
		10	34.59	0.11	35.27	0.17	36.52	0.51	35.46	0.91	100
		1	36.53	0.54	36.93	0.73	39.38	0.62	37.61	2.22	100

Table 6. Summary of analysis of covariance (ANCOVA) results for nine elephant species-specific reactions.

Reaction	Template Source	Source	DF	Sum of squares	Mean squares	F	Pr > F
Elephant Universal Fragment 1	Asian Elephant	Amount	1	4849.02	4849.02	246.02	< 0.0001
		Run	2	16.15	8.08	0.41	0.66
		Amount*Run	2	1.59	0.80	0.04	0.96
	African Elephant	Amount	1	5134.13	5134.13	221.29	< 0.0001
		Run	2	34.66	17.33	0.75	0.47
		Amount*Run	2	0.39	0.19	0.01	0.99
	Wooly Mammoth	Amount	1	6064.79	6064.79	206.56	< 0.0001
		Run	2	294.93	147.47	5.02	0.01
		Amount*Run	2	5.59	2.80	0.10	0.91
Elephant Universal Fragment 2	Asian Elephant	Amount	1	5066.24	5066.24	192.44	< 0.0001
		Run	2	28.44	14.22	0.54	0.58
		Amount*Run	2	1.48	0.74	0.03	0.97
	African Elephant	Amount	1	4415.72	4415.72	189.09	< 0.0001
		Run	2	10.60	5.30	0.23	0.80
		Amount*Run	2	7.97	3.99	0.17	0.84
	Wooly Mammoth	Amount	1	5689.52	5689.52	186.76	< 0.0001
		Run	2	22.87	11.44	0.38	0.69
		Amount*Run	2	2.79	1.40	0.05	0.96
Asian Elephant	Asian Elephant	Amount	1	4898.70	4898.70	189.62	< 0.0001
		Run	2	10.84	5.42	0.21	0.81
		Amount*Run	2	0.79	0.40	0.02	0.98
African Elephant	African Elephant	Amount	1	4756.73	4756.73	196.89	< 0.0001
		Run	2	24.76	12.38	0.51	0.60
		Amount*Run	2	6.39	3.20	0.13	0.88
Wooly Mammoth	Wooly Mammoth	Amount	1	4945.14	4945.14	213.85	< 0.0001
		Run	2	94.62	47.31	2.05	0.13
		Amount*Run	2	16.42	8.21	0.35	0.70

Table 7. Slopes and resulting reaction efficiencies and R^2 values for three runs of each elephant detection and species identification assay.

Reaction	Template Source	Run 1			Run 2			Run 3		
		Slope	Efficiency	R^2	Slope	Efficiency	R^2	Slope	Efficiency	R^2
Elephant Universal Fragment 1	Asian Elephant	-3.56	0.91	0.99	-4.06	0.76	0.99	-3.84	0.82	0.99
	African Elephant	-3.31	1.01	0.99	-3.50	0.93	0.99	-3.46	0.95	0.99
	Wooly Mammoth	-3.72	0.86	0.99	-3.71	0.86	0.99	-3.97	0.78	0.98
Elephant Universal Fragment 2	Asian Elephant	-3.47	0.94	0.99	-3.35	0.99	0.99	-3.68	0.87	0.99
	African Elephant	-3.26	1.03	0.99	-3.28	1.02	0.99	-3.46	0.95	0.99
	Wooly Mammoth	-3.38	0.98	0.99	-3.71	0.86	0.99	-3.67	0.87	0.99
Asian Elephant	Asian Elephant	-3.43	0.96	0.99	-3.37	0.98	0.99	-3.40	0.97	0.99
African Elephant	African Elephant	-3.11	1.09	0.99	-3.27	1.02	0.99	-3.43	0.96	0.99
Wooly Mammoth	Wooly Mammoth	-3.04	1.13	0.99	-3.19	1.06	0.99	-3.20	1.05	0.99

Table 8. Results of real-time PCR amplification of a dilution series of DNA from three elephant species, using initial elephant screening assays.

		Elephant Fragment 1		Elephant Fragment 2	
Species	Dilution	Estimated Copy Number	C_t	Estimated Copy Number	C_t
Asian	1/5	182670.3	22.9	188393.5	22.9
	$1/5^2$	39974.4	26.9	28089.9	26.0
	$1/5^3$	8366.0	28.9	6547.8	28.3
	$1/5^4$	1311.4	31.2	1109.6	31.1
	$1/5^5$	168.1	33.8	122.5	34.7
	$1/5^6$	25.9	36.1	12.8	36.8
	$1/5^7$	15.0	37.8	8.4	38.9
	$1/5^8$	6.4	39.0	0.0	Undetermined
	$1/5^9$	0.0	Undetermined	0.0	Undetermined
African	1/5	10551.1	26.3	10485.9	26.4
	$1/5^2$	2631.0	28.2	1612.7	29.0
	$1/5^3$	490.5	30.6	250.2	31.7
	$1/5^4$	59.4	33.6	43.0	34.2
	$1/5^5$	30.5	34.5	7.2	36.7
	$1/5^6$	1.8	38.5	0.6	40.2
	$1/5^7$	0.8	39.6	0.2	41.6
	$1/5^8$	0.0	Undetermined	0.0	Undetermined
	$1/5^9$	0.0	Undetermined	0.0	Undetermined
Mammoth	1/5	31.2	35.8	80.4	35.8
	$1/5^2$	7.1	37.9	17.5	38.3
	$1/5^3$	8.9	37.6	3.6	40.9
	$1/5^4$	3.2	39.0	4.0	40.7
	$1/5^5$	4.5	38.6	0.7	43.5
	$1/5^6$	0.0	Undetermined	0.0	Undetermined
	$1/5^7$	0.0	Undetermined	0.0	Undetermined
	$1/5^8$	0.0	Undetermined	0.0	Undetermined
	$1/5^9$	0.0	Undetermined	0.0	Undetermined

Table 9. Cost analysis of DNA sequencing and real-time PCR for species identification of 48 case samples.

	DNA Sequencing		Real-time PCR	
Method	Reagents	Technician time (rate of $25/hr)	Reagents	Technician Time (rate of $25/hr)
Extraction	$168.50	$50.00	$168.50	$50.00
Amplification - Traditional (sequencing) or Real-time	$37.10	$12.50	$314.96	$25.00
Agarose gel electrophoresis	$2.65	$50.00	NA	NA
PCR product cleaning	$84.27	$12.50	NA	NA
Sequencing reaction	$285.00	$25.00	NA	NA
Analysis and write up	NA	$200.00	NA	$50.00
Total	$577.52	$350.00	$483.46	$125.00
Total Cost Including Technician time	**$927.52**		**$608.46**	

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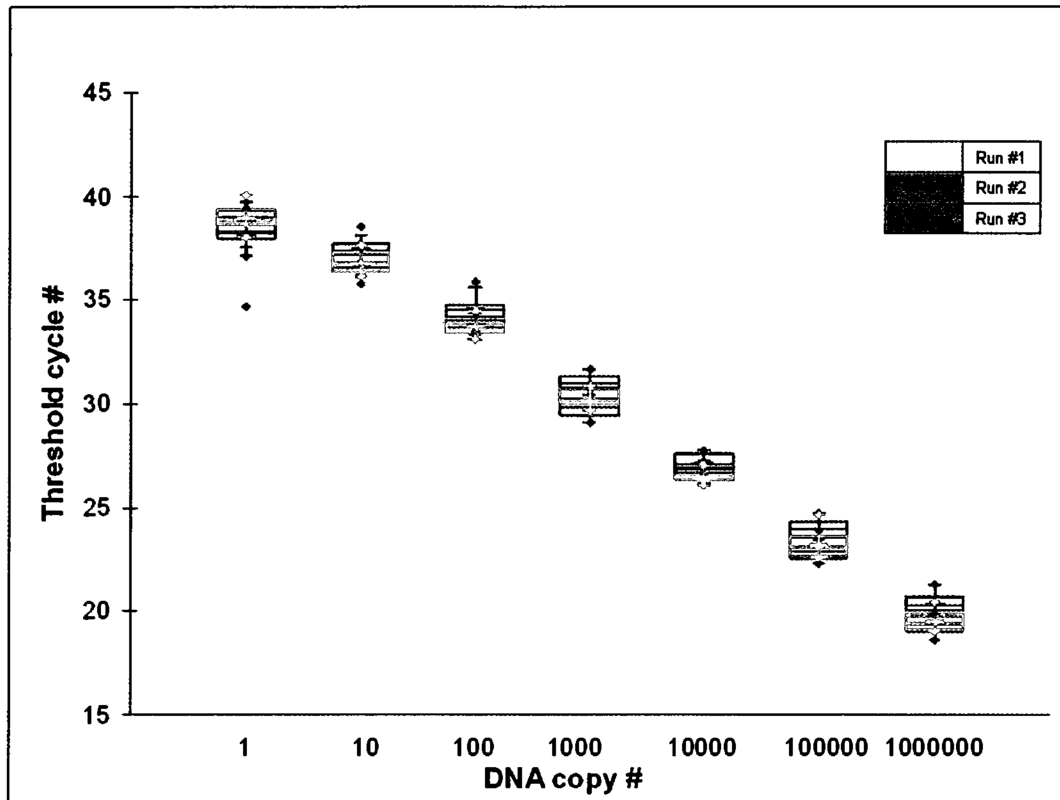
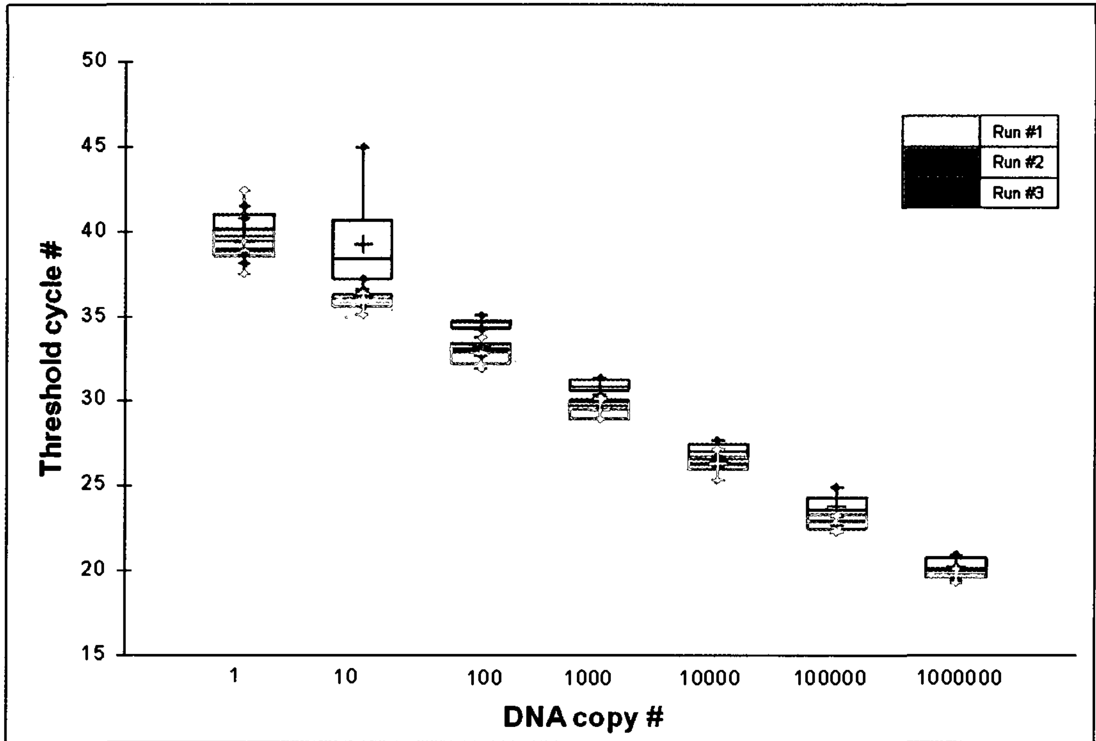
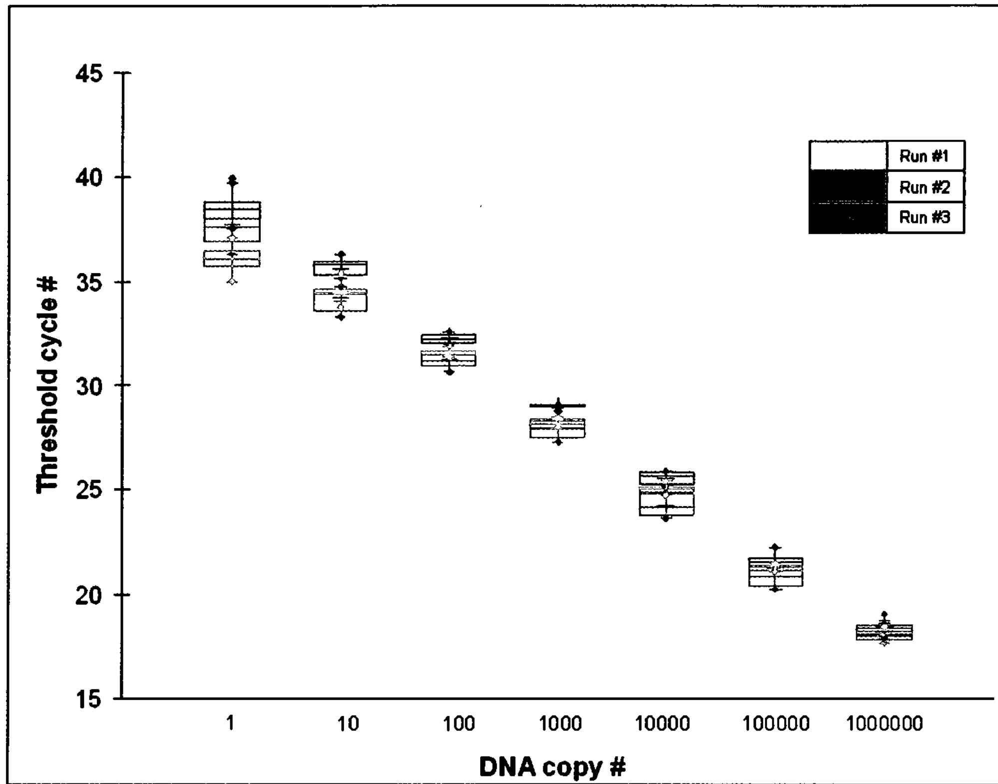


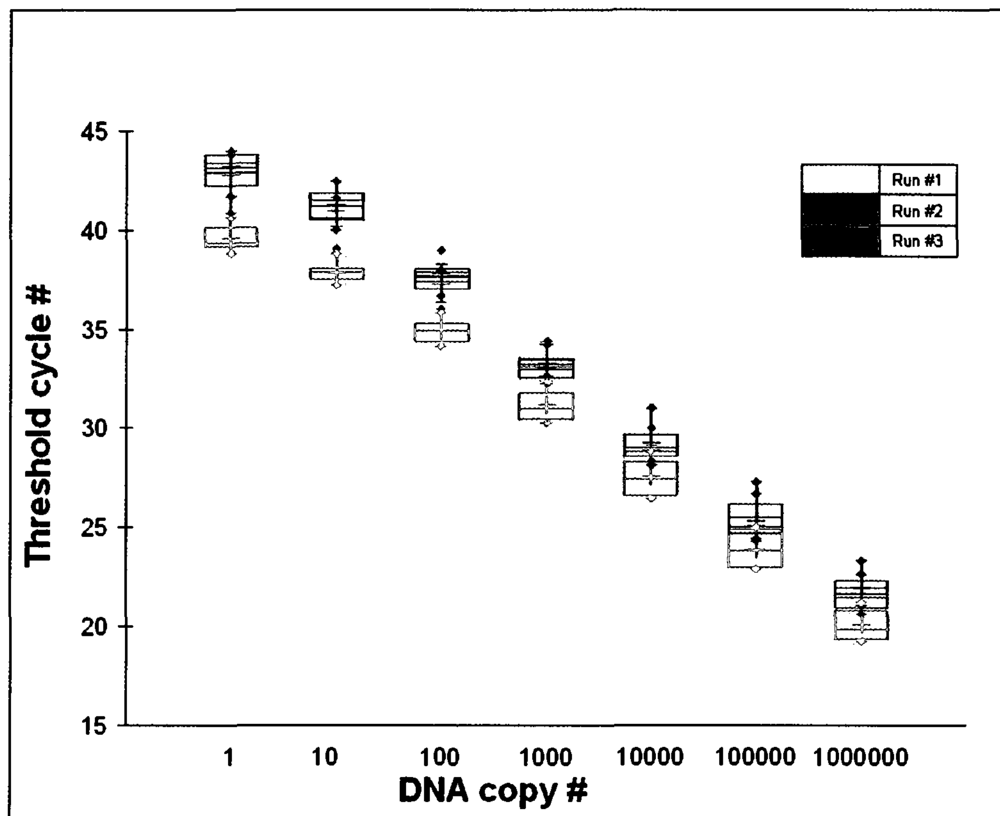
Appendix I. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.



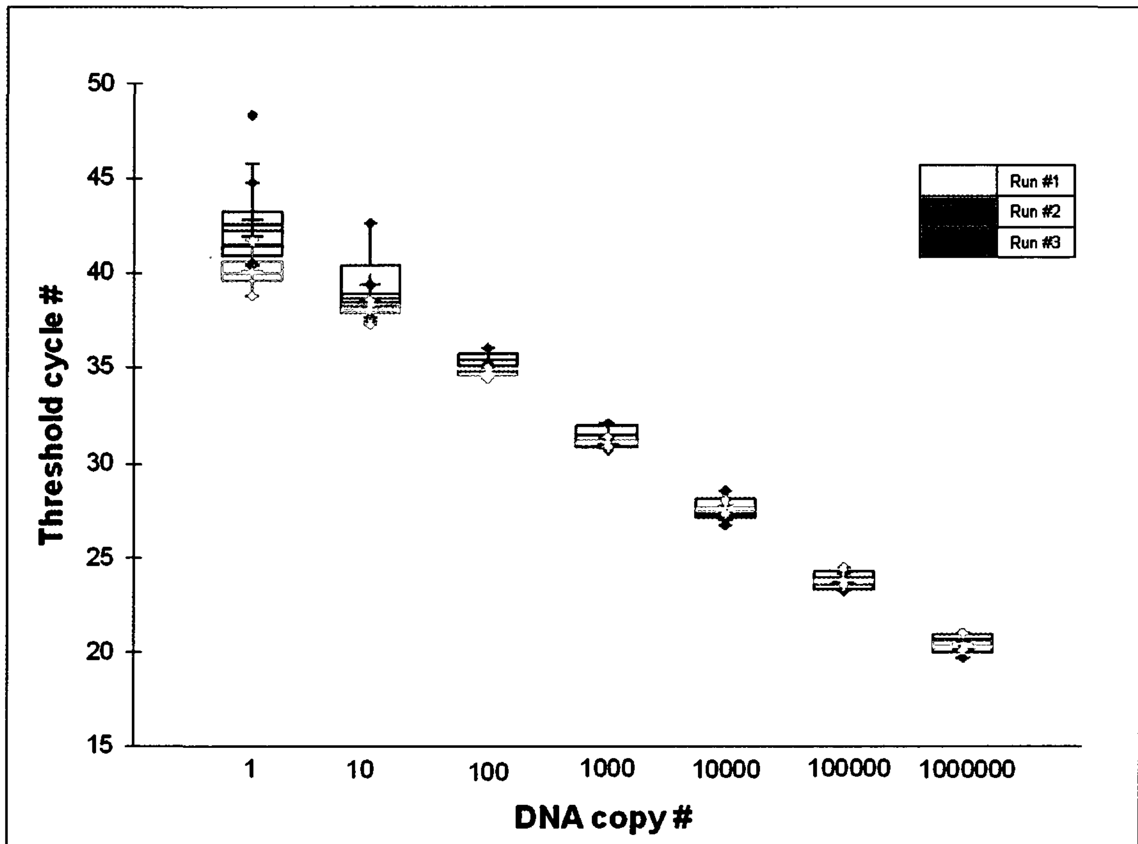
Appendix II. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.



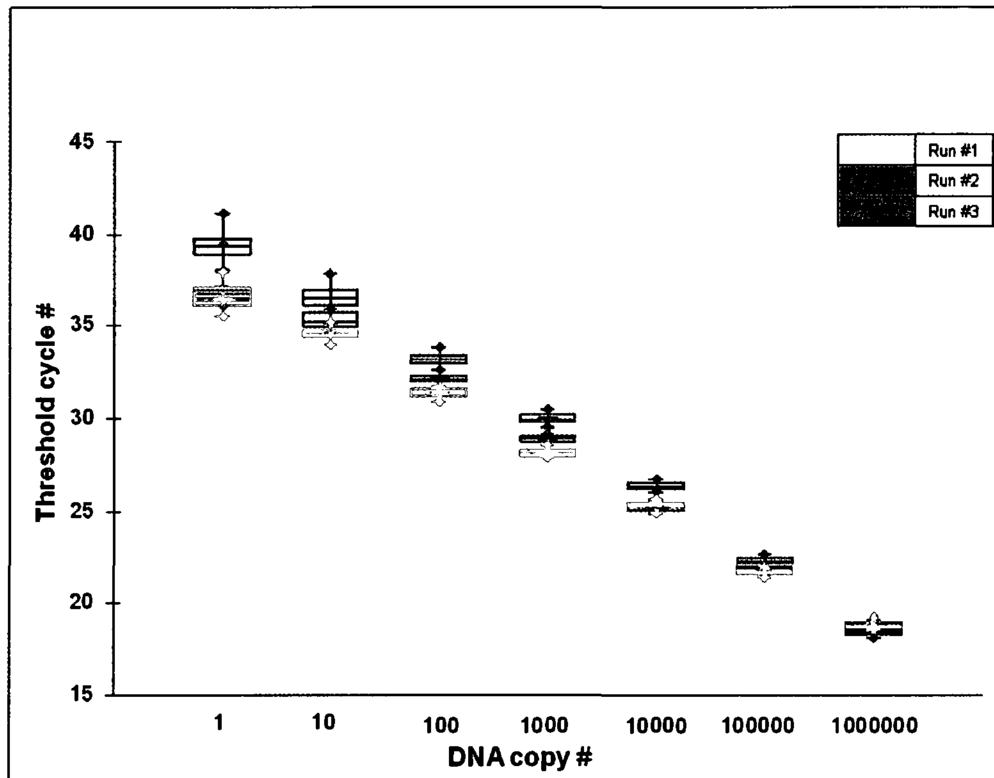
Appendix III. Box and whisker plots for three real-time PCR runs of African elephant species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of African elephant DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.



Appendix IV. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 1. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

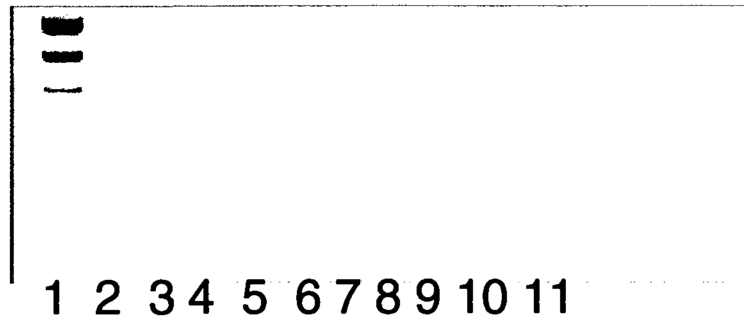


Appendix V. Box and whisker plots for three real-time PCR runs of elephant detection assay for fragment 2. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

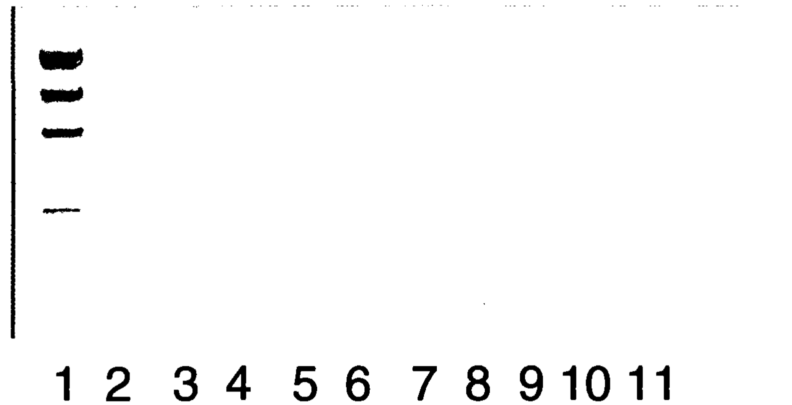


Appendix VI. Box and whisker plots for three real-time PCR runs of Woolly mammoth species identification assay. Box and whiskers show means, 1st and 3rd quartiles, 95% confidence limits and minimum and maximum values of Ct (threshold cycle number) for a 6 fold dilution series of Woolly mammoth DNA. Box and whiskers for each run represents data from 6 replicates per run for each template amount.

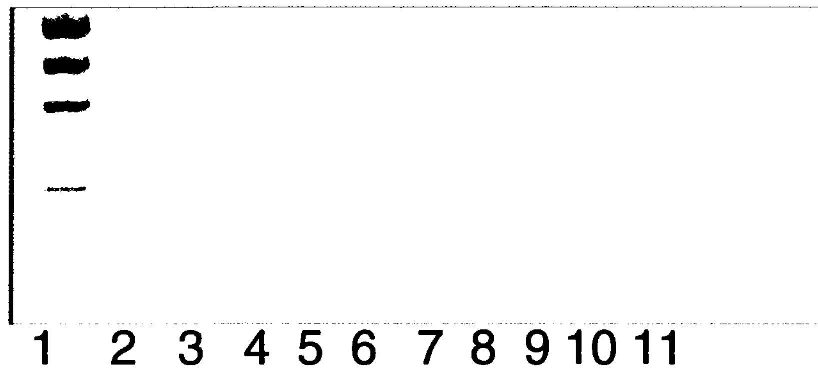
a)



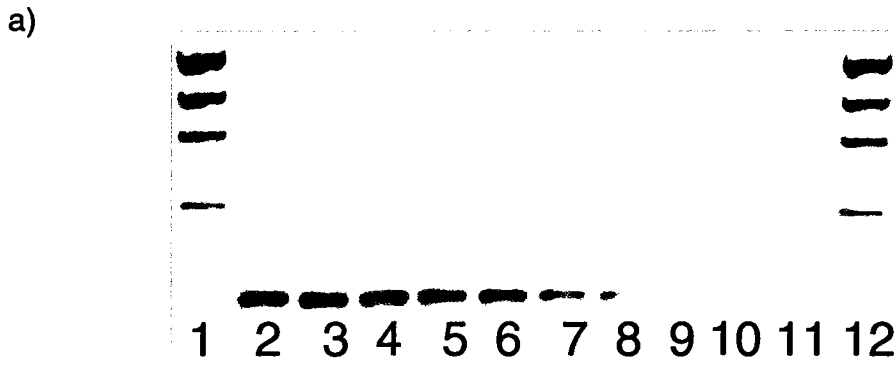
b)



c)



Appendix VII. a) Dilution series of African elephant stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
b) Dilution series of Asian elephant stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase. c) Dilution series of Woolly mammoth stock DNA amplified using fragment 1 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
From left; Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution, 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel.



Appendix VIII. a) Dilution series of Asian elephant stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase. b) Dilution series of Woolly mammoth stock DNA amplified using fragment 2 elephant universal primers and Ampli*Taq* Gold DNA polymerase.
From left; Lane 1) Low Mass DNA Ladder, 2) 1/5 dilution, 3) $1/5^2$ dilution, 4) $1/5^3$dilution, 5) $1/5^3$ dilution, 6) $1/5^5$ dilution, 7) $1/5^6$ dilution, 8) $1/5^7$dilution, 9) $1/5^8$ dilution, 10)$1/5^9$ dilution 11) reaction negative. All amplified products were stained with ethidium bromide and visualized on a 1.5% agarose gel.