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Real-time PCR detection and quantification of elephantid DNA: Species identification for highly processed samples associated with the ivory trade

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ABSTRACT

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 the international ivory trade. This assay is especially useful for highly processed samples where there are no distinguishing morphological features to identify the species of origin. Using species-specific *Taq*man® probes targeting a region of the mitochondrial cytochrome *b* gene, we developed an assay that 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), when positive identification of species from illegally traded products is required by conservation officers in wildlife forensic cases.

1. 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 [1,2]. 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 [3,4].

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. 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. 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).

Populations of African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephant species are currently listed under Appendix I or II of CITES, respectively, as many populations are highly endangered as a result of exploitation [5–7]. Recently DNA sequencing has shown that the forest elephant is a distinct species of elephant and not a sub species of the African elephant [8], however current CITES listings do not distinguish between these two types of elephants (www.cites.ec.gc.ca). Despite CITES regulations, the illegal trade of endangered species is a highly lucrative business generating billions of dollars in revenue worldwide [9]. For elephants there has been evidence of increasing illegal trade. Between August 2005 and August 2006 over 25 000 kg of ivory were seized worldwide, more than the combined total for the three years prior [10]. This works out to about 4000 elephants using an estimate of 6.6 kg of ivory/elephant [10] and it has been estimated that up to 8% of remaining African elephants are killed annually by poachers [10]. This death rate could mean the extinction of elephants in Africa by the year 2020 [10]. Female Asian elephant do not have tusks, and as a result male elephants are being targeted by poachers and as a result the selective harvest of males lay cause highly disproportionate sex ratios that will impact a population's

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ability to recover due to the inability to find a mate [7]. The ivory trade is recognized as the single most important cause of the decline in elephant populations worldwide [6].

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 [11]. This is the only extinct proboscidean species that consistently provides high quality, carvable ivory [12]. 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 [13]. Exports of mammoth ivory from Russia increased to 40 tonnes in 2007 from only 2 tonnes in 1989 [13]. There is evidence that poachers may be intentionally mislabelling elephant ivory as that of the extinct Woolly mammoth in order to avoid CITES regulations [14]. 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 [15]. Ivory is often carved into desirable shapes that 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.

The most widely accepted method of species identification in highly processed forensic case samples is DNA sequencing. DNA markers on the mitochondrial genome are most commonly used for animal species as they are more likely to be amplified compared to single copy nuclear loci in highly processed samples [16,17]. Sequencing of the cytochrome *b* region of the mitochondrial genome has been well established for use in the species identification of forensic samples [17–21] and may also provide additional information about a species and populations including phylogeny, divergence and haplotype information [21,22] 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 [17]. In addition, cytochrome *b* has been applied to forensic case work and has been used to identify species from case samples of meat [23] blood and hair [24], as well as from bones seized from traditional Chinese medicine traders [25], ivory from elephants [21,26], and horns from three species of rhinocerous [26].

While DNA sequencing is the most common assay for species identification of forensic samples, the process requires several steps and is relatively expensive [27,28]. DNA sequencing may also be affected by complex mixtures and by inhibitors found in many forensic samples [25]. 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. Lee et al. [21] observed poor sequence data in 2% of ivory samples which had shown evidence of amplification after a nested PCR. Poor sequence quality and ambiguous base calling may make it difficult to determine species in poor quality or low template samples.

In general it is difficult to obtain reliable evidence to assist in the prosecution of individuals of illegally trading endangered species [23,26]. This is especially true in cases where there are very small pieces of evidence and/or processing prevents morphological identification [26]. Positive identification of the species of origin should be obtained before prosecution is considered [25] and enforcement is often hampered by this lack of evidence [15].

Real-time PCR is a sensitive method that can be used to positively identify the presence of specific DNA fragments at very low quantities [29]. Real-time PCR measures the rate of amplification throughout the cycles as opposed to traditional PCR that requires the use of some type of end point analysis. A species-specific *Taq*man® (Applied Biosystems, Grove City, CA) 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 [29]. The resulting 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). 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 [29].

Real-time PCR has been used to positively identify small quantities of mtDNA from human peripheral blood and subcutaneous fat cells [30], to quantify mtDNA in forensic samples [31], to quantify both mtDNA and nuclear DNA in forensic samples and ancient human bone [32], as well as for species identification of tiger from blood samples [25]. 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 [33,34]. Real-time PCR has similarly been used to detect and quantify porcine, bovine, lamb, turkey, chicken and ostrich in complex samples [35].

We have developed a real-time assay that targets a 106 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 (*L. africana*), Asian elephant (*E. 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.

2. Methods

2.1. 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. 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 0.5 M EDTA (Invitrogen, Carlsbad, CA) 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 500 μl of lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroyl sarcosine, 10 mM 1,2-cyclohexanediaminetetraacetic acid, 0.1 M Tris–HCl,

pH 8.0). Each sample was incubated with 10 U of Proteinase K (Roche Diagnostics Corporation, Indianapolis, IN) at 65 °C for 2 h. 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, CA). DNA was eluted from the Qiagen column by adding 50 μl of 65 °C $TE_{0.1}$ (10 mM Tris, 0.1 mM EDTA).

2.2. Primer and probe design

All primers and probes were designed using the software Primer Express version 2.0 (Applied Biosystems, Grove City, CA). Primers and probes were designed from Genbank sequences of African elephant, Asian elephant, Woolly mammoth, Pygmy (forest) elephant, dugong, rhinoceros, hippopotamus and human that were aligned using Bioedit 6.0 [35]. Universal elephant probes and primers and species-specific probes were designed for Asian and African elephants and Woolly mammoth. The primers amplify a 106 base pair fragment of the cytochrome *b* gene initiating at base pair 14 983 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 three target 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 three target species.

Primer and probe sequences and fluorescent labels are listed in Table 1. 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. Universal primers are used in all species-specific assays except for the Woolly mammoth 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) with a fluorescent dye on the 5′ end and non-fluorescent quencher and minor groove binder on the 3′ end. The addition of a minor groove binder (MGB) will increase the melting temperature of the probe without increasing the length of the probe [37].

2.3. Development and optimization

A standard curve of mtDNA was developed through cloning our target fragment. 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 min in a water bath, then inserted into a bacterial vector following the protocol for gel-purified product in the TOPO cloning kit (Invitrogen, Carlsbad, CA). Single white colonies were picked for screening, placed in 50 μl $TE_{0.1}$ (Tris, 0.1 mM EDTA) and boiled for 15 min to lyse the bacterial cells and denature cellular proteins. Each clone was amplified using M13 primers from the TOPO cloning kit (Invitrogen, Carlsbad, CA) in a 100 μl reaction containing 1× PCR buffer, 0.2 mM of each dDNTP, 0.2 μM each primer, 0.5 units *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 2 μl 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, PA). Samples were run on a MegaBACE 1000 DNA Analysis system and analyzed with Sequence Analyser 3.0.

Sequences were aligned in Bioedit 6.0 [36]. 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 5 μl 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.

Table 1
Sequences and fluorescent labels for all primers and probes used in elephant species identification assays.

Name	Primer/probe	Sequence	5′ label
ElephantF	Primer	CCATCCTACGATCTGTACCAAACAAAC	None
ElephantR	Primer	CTTCGGTGCTTGGATGTATG	None
MammothR	Primer	CATACTTCGGTGTTTAGATGTA	None
Elephant	Probe	AGGCGTCCTAGCCCT	TET
African	Probe	CCTAGGATTAATACCACTTCT	6FAM
Asian	Probe	TGATTTTAGGATTAATACCATTTCT	VIC
Mammoth	Probe	CTAATCCTAGGAATTATACCACTTCTA	TET

For real-time PCR, a 20 μl reaction was prepared containing 5 μl of sample with a known quantity of mtDNA ranging from 10^6 copies to 1 copy, 1× PCR MasterMix and 0.3 μM each of forward primer, and 0.3 μM MGB probe (all Applied Biosystems, Foster City, CA), and remaining volume of sterile deionized distilled water (Invitrogen, Carlsbad, CA). DNA detection was performed using the Applied Biosystems 7900 sequence detection system. The reaction conditions for all assays were as follows; 10 min activation at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min 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.

2.4. 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 that can be worked in a similar fashion to ivory [12]. 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.

2.5. 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 [38] 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).

2.6. 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 20 μl volume using 1× PCR Gold Buffer, 1.5 mM $MgCl_2$, 2 mM of each dNTP (Invitrogen Life Technologies, Carlsbad, CA), 0.2 μM each primer, 1.25 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 5 μl of template DNA. Samples amplified using traditional PCR were run on a 1.5% agarose gel stained with ethidium bromide.

3. Results

3.1. 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. To develop mtDNA controls to be used in quantifications of unknowns against a standard curve, our target fragments were cloned by inserting amplified product into plasmid vectors. Benefits to using plasmids as standard controls include harvesting large amounts of standards, long-term stability and ease of quantification [39]. 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. 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.

Table 2
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 initial screening	Asian elephant species identification	African elephant species identification	Woolly mammoth species identification
Asian elephant 10^6	1 000 000.00	1 000 000.00	0.00	3.76
Asian elephant 10^5	100 000.00	100 000.00	0.00	0.88
Asian elephant 10^4	10 000.00	10 000.00	0.00	1.68
Asian elephant 10^3	1000.00	1000.00	0.00	0.45
African elephant 10^6	1 000 000.00	0.00	1 000 000.00	0.38
African elephant 10^5	100 000.00	0.00	100 000.00	0.84
African elephant 10^4	10 000.00	0.00	10 000.00	0.62
African elephant 10^3	1000.00	0.00	1000.00	0.94
Woolly mammoth 10^6	1 000 000.00	0.00	0.00	1 000 000.00
Woolly mammoth 10^5	100 000.00	0.00	0.00	100 000.00
Woolly mammoth 10^4	10 000.00	0.00	0.00	10 000.00
Woolly mammoth 10^3	1000.00	0.00	0.00	1000.00

3.2. 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 results of specificity testing for each assay on all elephant species are summarized in Table 2. We tested ten-fold dilutions of standards ranging from 10^6 copies to a single copy of elephant mtDNA per reaction. In the initial screening reaction 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.

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 and could be ignored in all cases.

3.3. 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 Fig. 1. 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 Fig. 2. 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 Appendix I. Samples with 10 copies amplified 99% of the time and

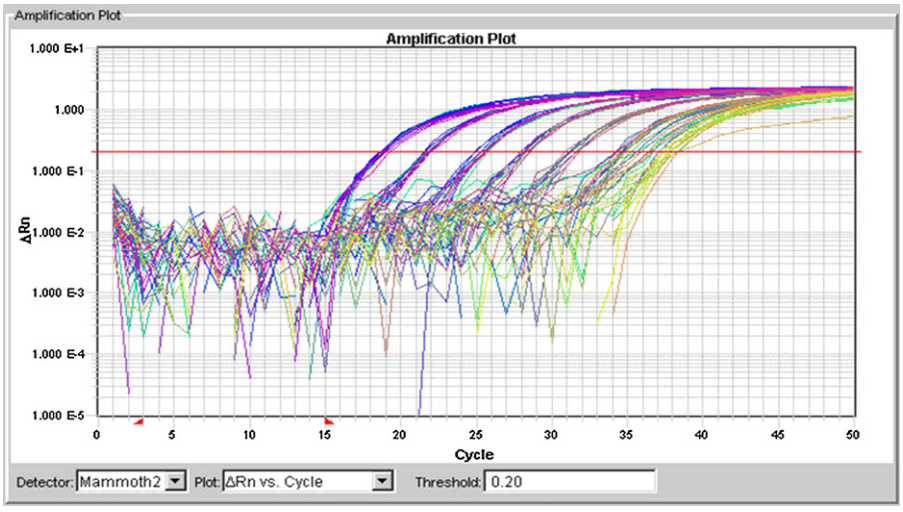

Fig. 1. Amplification plot of dilution series of Woolly mammoth DNA amplified using species-specific reaction. The threshold cycle is indicated by the horizontal red line. Samples with 10^6 copies of template cross the threshold first around cycle 19, followed by 10^5, 10^4, 10^3, 10^2, 10 and 1 copy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

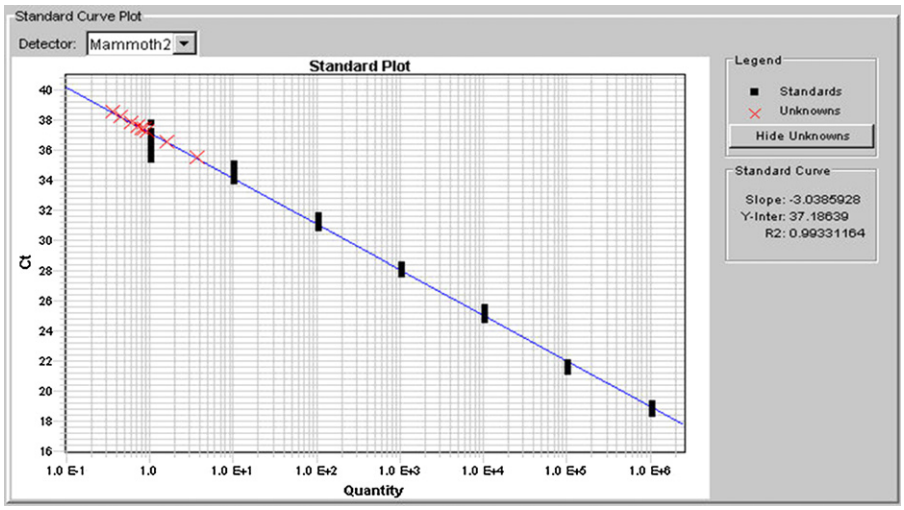

Fig. 2. Standard plot of threshold cycle and DNA template amount for a 6-fold dilution series ranging from 1 million to 1 copy of Woolly mammoth mitochondrial DNA, amplified in species-specific reaction. The resulting standard curve can be used for quantification of Woolly mammoth DNA in unknown samples.

those with only a single copy amplified 90% 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. The results of statistical testing with ANCOVAS showed 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. Further analysis of the ANOCOVA results with a Tukey test showed that there was a significant difference in all pairwise comparisons of template amount.

3.4. Evaluation of sensitivity on control DNA

To evaluate sensitivity we considered traditionally amplified samples that exhibited at least 15 ng 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, PA). 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.

4. Discussion

Despite international regulations the trade of ivory and elephant products remains a threat to the world's elephant populations [6]. The aim of this study was to design and validate a highly sensitive DNA based assay that 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. 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 [40].

An increase in the variability in detecting low template amounts has also been observed in other studies. For quantification of human mtDNA by von Wurmb-Schwark et al. [31], 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. Lopez-Andreo et al. [35] observed a loss of linearity in their standard curve for samples showing results above 40 cycles. A PCR of more than 40 cycles can increase the amount and complexity of non-specific background products [41]. 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 consistent and reproducible results and detectable fluorescence before 40 cycles had been completed.

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 [31] or variability associated with dilution and pipetting of low quantities of DNA. Our success rate for single copies of 90% across assays is comparable to others. Wetton et al. [25] reported a 63% success rate with single molecules and 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 [32]. The success rate observed for samples with a single copy is an important factor in determining the quality of each reaction. A decrease in sensitivity indicates a problem and therefore 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 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. [42] observed an increase in detection sensitivity as compared to iso-electric focusing (IEF) method for egg species identification; Alonso et al. [32] 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. [40] 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, WI) 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. 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. 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. Lee et al. observed a 2% failure rate in confiscated ivory samples due to poor quality sequence data in samples which showed evidence of amplification [21], and similar failure rate could be expected for our amplified samples. 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 [43].

When mtDNA sequence evidence is used in court all of the above issues may be raised as possible reasons to exclude it. In contrast, the use of real-time PCR can 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 sequencing requires 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. For this reason real-time PCR should be chosen over DNA sequencing for forensic cases that simply require positive species identification.

Another benefit to real-time PCR is the cost effectiveness. 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, and in some cases reamplified for nested PCRs [20,21], 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. An analysis would confirm that control samples amplified as expected with a linear standard curve and a report would be written.

Reducing the number of processing steps in an assay is beneficial with respect to forensic casework. 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.

A recent assessment of genomic DNA of both living and extinct elephant species by Rohland et al. concluded that the forest elephant is a distinct species of elephant and not a sub species of the African elephant as has been previously thought [8]. At the present time the CITES listing for forest elephants do not reflect this updated phylogeny and this form remains listed as a subspecies (www.cites.ec.gc.ca). If the listing of forest elephant should change to include this form as a distinct species this assay would need to be updated to include an additional test using a species specific probe for forest elephants. The new assay should be performed on those samples which have been determined to be African elephant using the current methodology.

5. Conclusion

The real-time PCR technique enables rapid and cost-effective identification for both CITES listed elephant species and the extinct Woolly mammoth. 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.

The impact of CITES regulations 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.forsciint.2011.12.006.

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