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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,