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# A novel real time PCR assay using melt curve analysis for ivory identification

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ABSTRACT

Demand for ivory and expansion of human settlements have resulted in a rapid decline in the number of elephants. Enforcement of local and international laws and regulations requires identification of the species from which any ivory, or ivory products, originated. Further geographical assignment of the dead elephant from which the ivory was taken can assist in forensic investigations. In this study, a real-time PCR assay using melt curve analysis was developed and fully validated for forensic use. The presence or absence of three Elephantidae-specific and elephant species-specific melting peaks was used to identify the elephant species. Using 141 blood and ivory samples from the three extant elephant species, the assay demonstrated very high reproducibility and accuracy. The limit of detection was as low as 0.031 ng of input DNA for conventional amplification and 0.002 ng for nested amplification. Both DNA concentrations are typically encountered in forensic casework, especially for degraded samples. No cross-reactivity was observed for non-target species. Evaluation of direct amplification and nested amplification demonstrated the assay's flexibility and capability of analyzing low-template DNA samples and aged samples. Additionally, blind trial testing showed the assay's suitability application in real casework. In conclusion, wildlife forensic laboratories could use this novel, quick, and low-cost assay to help combat the continuing poaching crises leading to the collapse of elephant numbers in the wild.


## 1. Introduction

Demand for ivory and expansion of human settlements have resulted in a rapid decline in the number of elephants [1]. An effort to prevent their extinction was made in 1989 by the Convention on International Trade in Endangered Species of Flora and Fauna (CITES), which lists all extant elephant species in CITES Appendix I. However, in 1997 and 2000, four African elephant populations (Botswana, Namibia, Zimbabwe, and South Africa) were listed in the Appendix II, which allows regulated trading of their ivory [1]. The change initiated and resulted in further poaching [2]. It is evident by the continuous seizures of ivory and the rise in illegally killed elephants in the past 15 years [1]. In fact, one of the largest-ever ivory destruction (105 tons from over 7000 elephants) was carried out by the Kenyan government early in 2016 [3]. African elephant ivory is often smuggled to black market hotspots such as Thailand and China where trading of the local Asian species is permitted [1]. Therefore, the African elephant ivory is laundered and traded as Asian elephant ivory.

Species identification is a common practice in wildlife crime investigation. Evidence needs to be demonstrated that a seized sample originated from a protected species in order to enforce national legislations. Three elephant species – Asian elephant (*Elephas maximus*), African bush elephant (*Loxodonta africana*), and African forest species (*Loxodonta cyclotis*) – are recognized at present based on morphology and genetics [4–6]. At ivory trade hotspots such as China, Thailand, and Malaysia, species identification is necessary to discriminate local Asian ivory from illegal African ivory, which is illegal. A number of methods have been proposed, including morphology-based macroscopic identification, short-wave near-infrared spectroscopy, and Fourier-transformed Raman spectroscopy [7,8]. These methods are non-destructive and provide both qualitative and quantitative data; however, there are many reasons that prevent them from being

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used in routine forensic investigations. These reasons include the inability to identify the elephant species from which the ivory was taken; expensive equipment; high false positive and negative rates; and lack of robustness for use with highly degraded or processed samples commonly found in crime scenes [7–10].

A molecular approach can overcome these drawbacks and provide greater confidence in species assignment. Molecular markers have been designed based on mitochondrial DNA loci such as cytochrome *b* (cyt *b*) and the control region using either standard or nested polymerase chain reaction (PCR) [11–13]. Significant disadvantages of the PCR-sequencing combinations include: being expensive and time-consuming; requiring a moderately long, intact DNA; cannot be used to analyze mixed DNA samples; and not being suitable for highly degraded samples.

Instead of DNA sequencing, a real-time PCR assay for elephant species identification can provide both quantitative and qualitative information, with the added benefits of being highly sensitive and less prone to errors from post-PCR processes. Wozney and Wilson [14] developed a *TaqMan*-based real-time PCR assay for such purpose. Compared to *TaqMan* probes, the use of an intercalating dye in combination with melt curve analysis is cheaper and up to a few targets can still be multiplexed. Melt curve analysis has been widely applied for the identification of many species, subspecies, and even for body fluid analysis (e.g. [15–17]).

Real-time PCR with melt curve analysis has never been applied to elephant species identification. In this study, we aimed therefore to develop and validate the first fully functional real-time PCR assay based on the use of melt curve analysis of both Elephantidae- and elephant species-specific SNPs for a sensitive and accurate identification of the elephant species from which the ivory was taken. The assay developed in this study should be beneficial to wildlife forensic laboratories and those involved in combating wildlife crime.

## 2. Materials and methods

### 2.1. Sample collection

A total of 141 samples comprising 52 samples from Asian elephant (*Elephas maximus*), 62 samples from African bush elephant (*Loxodonta africana*), and 27 samples from African forest species (*Loxodonta cyclotis*) were collected from the DNP Wildlife Forensic Unit, Department of National Parks, Wildlife and Plant Conservation, Thailand and Songkhla Zoo, Thailand (Table 1). Samples were in the form of liquid blood or confiscated ivory. For liquid blood, species were confirmed by a veterinarian using gross morphological characteristics prior to drawing blood. These blood samples were collected by a veterinarian and kept in a vacutainer tube containing ethylenediaminetetraacetic acid (EDTA). To confirm the identity of the species from which the ivory samples came, PCR with universal *cyt b* primers followed by sequencing was performed prior to this study [18]. Ivory was incised from the inside of a proximal-hollow ivory called dentin then cut into small pieces, using a sterile scalpel and bone scissors. They were then kept separate in sterile plastic bags until further analysis.

**Table 1**
Details of elephant species, type of sample, sample number, and where they were collected from. DNP = DNP Wildlife Forensic Unit, Department of National Parks, Wildlife and Plant Conservation, Thailand. SZ = Songkhla Zoo, Thailand.

| Elephant species | Sample type | Source | No. of samples |
|---|---|---|---|
| *Elephas maximus* | Confiscated ivory | DNP | 2 |
| | Blood | SZ | 50 |
| *Loxodonta africana* | Confiscated ivory | DNP | 62 |
| *Loxodonta cyclotis* | Confiscated ivory | DNP | 27 |
| Total | | | 141 |

### 2.2. DNA extraction

Tiny pieces of ivory samples were placed in a 1.5 mL microcentrifuge tube until the tube was half-filled. The samples were decalcified using 700 μL of 0.5 M EDTA. The solution was left for seven days or until the sample softened before being scraped using a sterile scalpel. The scraped ivory sample was then transferred to a new 1.5 mL microcentrifuge tube and 20 μL of proteinase K (10 mg/mL) was added. The solution was incubated at 56 °C for 24 h and centrifuged at 11,000 rpm for 1 min. The supernatant was then used for DNA extraction using the Favorgen Stool Kit (Favorgen Biotech Corporation, Taiwan) while DNA from blood sample was extracted using the QIAamp DNA Mini Kit (Qiagen, UK), according to the 'Blood' protocol. The DNA extracts were stored at −20 °C until further analysis.

### 2.3. Direct PCR sample preparation

Direct amplification of elephant blood and ivory samples were adapted from Kitpipit et al. [19,20]. Tiny pieces of ivory samples were placed in a 1.5 mL microcentrifuge tube and soaked in 20 μL PBS for two min. The solution was then incubated at 98 °C for a further two min. From the PBS solution, 1.5 μL was added directly to the qPCR reaction mix.

### 2.4. Elephantidae- and elephant species-specific SNPs identification

To identify Elephantidae-specific SNPs, 10 sequences from each elephant species and 207 sequences from 147 mammalian species covering broad taxonomic groups were aligned using Mega 5 [21]. SNPs that were specific to Elephantidae and not present in other mammalian species were identified manually.

To identify elephant species-specific SNPs, all elephant cyt*b* and ND5 gene sequences available on GenBank at the time of the study were downloaded. A total number of 1403 sequences, comprising 483 from *E. maximus*, 779 from *L. africana*, and 141 from *L. cyclotis*, were obtained. These sequences were then aligned using the program Mega 5 [21]. Among these sequences, 14 (one from *E. maximus*, 11 from *L. africana*, and two from *L. cyclotis*) were found to contain several anomalies assumed to be DNA sequence errors and were removed from further analysis. The SNPs or nucleotide bases that were specific only to one elephant species were located manually.

### 2.5. Primer design

Elephantidae- and elephant species-specific primers were designed by designing the target SNPs to be at the last base of the 3′-end of the primers. The candidate primers were checked for their physical parameters such as annealing temperature (Ta), GC content, primer length, and secondary structures using the bioinformatics web-based tool Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). Primers were ordered from Macrogen Inc., Korea. uMELT bioinformatics tool was used to predict and select for PCR products that have sufficiently different melting temperatures to enable unambiguous species identification [22]. Table 2 shows the primers used in the optimized assay.

### 2.6. Real-time PCR amplification

#### 2.6.1. Conventional amplification

Singleplex and multiplex real-time amplification and melt curve analysis were performed using a CFX96™ real-time PCR