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ORIGINAL ARTICLE

Ivory identification by DNA profiling of cytochrome b gene

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Abstract Ivory can be visually identified in its native form as coming from an elephant species; however, determining from which of the three extant elephant species a section of ivory originates is more problematic. We report on a method that will identify and distinguish the protected and endangered elephant species, *Elephas maximus* or *Loxodonta sp.* To identify the species of elephant from ivory products, we developed three groups of nested PCR amplifications within the cytochrome b gene that generate amplification products using highly degraded DNA isolated from confiscated ivory samples dating from 1995. DNA from a total of 382 out of 453 ivory samples were successfully isolated and amplified leading to species identification. All sequences were searched against GenBank and found to match with *E. maximus* and *Loxodonta sp.* with at least 99% similarity. The samples that were tested came from eight Asian elephants, 14 African forest elephants (*Loxodonta cyclotis*), and 360 African savannah elephants (*Loxodonta africana*). This study demonstrates a high success rate in species identification of ivory by a nested PCR approach within the cytochrome b gene which provides the necessary information for the protection of endangered species conservation.

Keywords Ivory identification · Elephant · Cytochrome b · Nested PCR · *Elephas maximus* · *Loxodonta cyclotis* · *Loxodonta africana*

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Introduction

The Asian (*Elephas maximus*) and the two African elephants (*Loxodonta cyclotis and Loxodonta africana*) are the three extant species of Elephantidae. *E. maximus* and *L. africana* are listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; *L. africana* is on Appendix II for Botswana, Namibia, South Africa, and Zimbabwe; see http://www.cites.org/eng/app/appendices.shtml). The trade in ivory is the main reason for the decline in numbers of these three species and it is the identification of the species from which the ivory originates that is required for the enforcement of CITES regulations. Asian and African

elephant ivory share very similar morphology making then difficult to differentiate visually. Several non-destructive methods, such as using variable wavelengths of light including visible, short-wave near-infrared, and Fourier-transform Raman spectroscopy have been developed [1, 2]. Analysis for the identification of ivory using organic compounds is possible. More recently, X-ray fluorescence, inductively coupled plasma atomic emission spectroscopy, inductively coupled plasma mass spectroscopy, and isotopic ratio mass spectrometer were used to analyze elemental and isotopic ratios in elephant ivory [3]. The qualitative and quantitative information obtained from these analyses is valuable but they can be influenced by environmental factors.

The molecular analysis of genetic material is another useful method to identify the species of illegal imported ivories and ivory-like materials. Mitochondrial loci such as the D-loop, cytochrome b (cyt b), 12S rDNA, and 16S rDNA have been used in phylogenetic studies, forensic application, and species identification of animals [4–8]. These loci along with autosomal microsatellite loci were also used in the related study of elephants [9–12]. A total of 16 autosomal microsatellite loci have been characterised to determine the geographic origin of African elephants [13, 14]. In these studies, it is reported that ivory samples have lower success rates (about 55%) of obtaining a polymerase chain reaction (PCR) product compared to tissue and dung. It is expected that the DNA within ivory will be severely degraded limiting the amount and quality of DNA that can be extracted. In previous reports, the method of nested PCR has been used to amplify hair samples [15, 16] and human remains [17] and also used successfully in species identification of rhinoceros horns, shahtoosh wool, and tortoise shells in our previous studies [18–20]. In this study, we report on the identification of 453 seized ivory samples dating from 1995 using a novel series of nested primers on the cyt b gene.

Materials and methods

DNA sources

A total number of 453 confiscated ivory samples were provided by the Council of Agriculture (COA), Taiwan. Blood stain samples from two Asian elephants (*E. maximus*) and two blood stains from African savannah (*L. africana*) elephants were provided by Taipei Zoo as reference samples. After pulverising the ivory sample with a sterilised file, approximately 50 mg of ivory powder were suspended in extraction buffer (0.1 M Tris–HCl pH 7.5, 3% sodium dodecyl sulfate, 60 mM NaCl) with 10 μg/μl of proteinase K and incubated at 56°C overnight. DNA was extracted with the modified salt–chloroform method [20]. A negative control using 50 μl of ddH_2O in place of the ivory was co-extracted with the ivory samples. The resulting DNA was dissolved in 30 μl of ddH_2O. DNA extraction of reference blood stains was performed with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Nested PCR amplification of partial cytochrome b gene and DNA sequencing

Three groups of nested PCR amplification were adopted in this study. The primer sequences and primer pairs of nested PCR are shown in Supplementary Tables 1 and 2. The universal primers of L14724 and H15149 were designed according to the report by Irwin et al. [21]. Primers H15197 and L14696 were designed according to our previous study [18] and the primers of L14768, H14957, and H15134 were designed according to the sequences of elephant species in GenBank. The outer primer pair was used first in the PCR amplification. If the resulting PCR products were insufficient for DNA sequencing, a second PCR was performed using the inner primer pair and 1 μl of the first PCR products as template. PCR amplification was performed in a 50-μl reaction mixture, which contained 5 μl of genomic DNA (5 μl of ddH_2O was added to a PCR negative control), reaction buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% (*w*/*v*) gelatin and 0.1% TritonX-100), 0.15 μM each of primers, 100 μM deoxynucleotide triphosphate, and 1.25 units of VioTaq DNA polymerase (Viogene, Taipei, Taiwan). The amplifications were conducted in a 2400 Perkin-Elmer thermal cycler with the following conditions: 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 45 s. PCR products were checked on a 2% agarose gel, purified with the PCR-M™ Clean Up System (Viogene), sequenced using the forward and reverse primers and the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The products were analyzed with POP-7™ (Applied Biosystems, Foster, CA, USA) and detected by ABI 3730 DNA Analyzer.

Sequence analysis

DNA sequences were aligned using the PileUp program and subjected to a similarity search using the Fasta program of the GCG computer package. The genetic distance analysis was generated by Kimura's two-parameter and a phylogenetic tree was constructed by the neighbor-joining method of the Phylip computer package. The GCG and Phylip computer package are available from http://bioinfo.nhri.org.tw (National Health Research Institute, Taiwan).

Results and discussion

The strategy of nested PCR amplification

In order to obtain the longest PCR amplicons possible, PCR amplifications for each sample were performed from groups 1 to 3 (Supplementary Table 2) in order. When a PCR product suitable for DNA sequencing was obtained, no further PCR was performed. Of the 453 samples, 156 were successfully identified using the largest nested primer sets producing an amplicon of 486 bp that were amplified successfully and sequenced using a nested secondary PCR primer set. The intermediate primer set successfully identified 33 samples and 193 samples were successfully identified using the smallest nested primer sets (Supplementary Table 2). There were 71 ivory samples that failed to be identified by these strategies. Of these, 63 samples produced no detectable PCR product after the nested amplification and the other eight samples produced a PCR product but no meaningful DNA sequence data could be obtained.

To identify the presence of any contamination, the DNA extraction and PCR negatives were used. No detectable PCR product was obtained from any negative control. The reference samples were examined in a different laboratory to minimize contamination and it is one of the advantages when working on trace nonhuman DNA that the laboratory staff cannot contaminate the samples.

In this study, there were 382 ivory samples that could be successfully amplified and sequenced using the three strategies of nested PCR amplification. The 84.3% success rate indicates the benefit of this strategy of nested PCR amplification when analyzing highly degraded DNA.

Sequence analysis

The sizes of sequencing results were 486, 405, and 228 bp from the three nested PCR products. After excluding the primer sequences and sequences upstream of the first codon on the cyt b gene, fragments of 402, 365, and 188 bp were selected for sequence comparison (Supplementary Table 2). The 160 samples (including four reference samples) that produced a 402 bp fragment resulted in 43 variable sites which showed 11 haplotypes (Table 1). The fragment of 365 bp was located between nucleotide positions 23 and 387 of the cyt b gene and there were 38 variable sites identified within the 193 samples representing 11 haplotypes. The fragment of 188 bp was located between nucleotide positions 23 and 210 of the cyt b gene and there were 21 variable sites representing nine haplotypes within all of the 386 samples successfully analyzed (including four reference samples). These nine haplotypes are shown in Table 1 where types 6, 7, and 8 had the same sequence based on the 188-bp fragment but differed if using the 402 bp data. The distribution of the variable sites in the 402, 365, and 188 bp fragments were 10.70%, 10.41%, and 11.17%, respectively.

Species identification

The DNA sequences of 11 haplotypes generated from the 402 bp, along with the 11 haplotypes from the 365 bp and

Table 1 Polymorphic sites and haplotypes with the 402 bp fragment of the cytochrome b region from 156 ivory samples and 4 blood stain reference samples

Haplotype	N[b]	Nucleotide position[a]																																								
																	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	4
		1	1	2	2	2	2	3	3	3	4	6	6	7	8	9	1	3	4	4	6	6	7	0	0	1	1	4	6	6	7	7	0	1	2	3	3	4	4	4	5	6	9	0
		1	2	0	4	7	8	0	1	3	5	6	9	5	5	6	7	2	5	7	2	5	1	1	7	6	9	6	4	7	0	3	0	5	1	0	6	2	5	2	5	6	6	2
E. maximus[c]	2	C	C	T	C	C	C	G	T	T	C	C	C	T	A	T	A	A	T	A	T	C	T	C	C	C	C	A	T	T	C	C	A	A	C	A	C	T	A	C	T	T	C	A
Type 1	8	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	C	T	.	.	.	T	.	.	C	.	T	C	C	.	.
L. africana[c]	2	T	T	C	.	.	T	A	C	.	T	T	.	C	.	.	.	.	.	.	.	T	C	.	T	T	G	.	C	.	.	.	.	G	.	G	T	.	C	.	.	C	.	G
Type 2	2	T	T	C	T	T	.	A	C	.	T	T	T	C	G	.	.	G	C	G	C	T	C	.	.	T	A	.	.	.	.	.	G	.	.	G	.	.	C	.	.	C	T	G
Type 3	1	T	T	C	T	T	.	A	C	C	T	T	.	C	G	.	.	G	C	.	C	T	C	.	.	T	A	.	.	.	.	.	G	.	.	G	.	.	C	.	.	C	.	G
Type 4	8	T	T	C	T	T	.	A	C	C	.	T	.	C	G	C	.	G	C	.	C	T	C	.	.	T	A	.	.	.	.	.	G	.	.	G	.	.	C	.	.	C	.	G
Type 5	3	T	T	C	T	T	.	A	C	C	.	T	.	C	.	.	.	G	C	.	.	T	C	.	.	T	A	.	.	.	.	.	G	.	.	G	.	.	C	.	.	C	.	G
Type 6	34	T	T	C	.	.	T	A	C	.	T	T	.	C	.	.	.	G	.	.	.	T	.	.	T	T	G	.	C	.	.	.	G	G	.	G	T	.	C	.	.	C	.	G
Type 7	97	T	T	C	.	.	T	A	C	.	T	T	.	C	.	.	.	G	.	.	.	T	.	.	T	T	G	.	C	.	.	T	.	G	.	G	T	.	C	.	.	C	.	G
Type 8	1	T	T	C	.	.	T	A	C	.	T	T	.	C	.	.	.	G	.	.	.	T	.	.	T	T	G	.	C	.	.	T	.	G	.	.	T	.	C	.	.	C	.	G
Type 9	2	T	T	C	.	.	T	A	C	.	T	T	.	C	.	.	.	G	.	.	.	T	.	T	T	T	G	.	C	.	.	T	.	G	.	G	T	.	C	.	.	C	.	G

[a] The code starts from the first base of cyt b gene
[b] Sample size
[c] Reference sample

nine haplotypes from the 188 bp fragment, were subjected to a similarity search using the Fasta program. All of the different haplotypes have been confirmed by independent experiments from pulverization of the ivory through to sequence analysis. The result of the Fasta search of the 11 sequence haplotypes from the 402 bp fragment, the type 1 haplotype from Table 1, and the reference sample Asian elephant matched the sequence of accession number D50846 and D83048 of *E. maximus* with a similarity of 100%, respectively. Types 2 to 5 haplotypes matched *L. cyclotis* (African forest elephant) with a similarity of 99.8% to 100%. Haplotypes 6–9 and the reference sample African savannah elephant matched *L. africana* with a similarity of 99.8% to 100%. The search results of the sequence types of the 365 and 188 bp resulted in a similar tendency as those using the 402 bp fragment. The result of the Fasta search using the 382 samples indicated that eight samples (type 1) belonged to Asian elephant (Table 1). The remaining 374 samples all came from one of the African elephant species with 14 matching the forest elephant and 360 matching the savannah elephant. Our study has shown that confiscated ivory in Taiwan comes predominantly from the two African elephant species of which the savannah elephant is the most common.

Genetic distance and cluster analysis were performed to estimate the efficiency of species identification using the partial sequence of the cyt b gene within the 402, 365, and 188 bp fragments. All of the sequence types identified in this study (Table 1), AY397664 of *E. maximus*, AY359274 of *L. cyclotis*, and D84151 of *L. africana* accessed from GenBank, formed part of the cluster analysis. Accession number NC 009574 of *Mammut americanum* in the GenBank database sequence was selected as an out group [22]. This study comprised 360 (94.2%) confiscated ivory samples shown to belong to *L. africana* but the intraspecies genetic diversity of this African savannah elephant is lower compared to the other two species based upon the fragment of 402 and 365 bp within the cyt b gene (Supplementary Table 3). These findings are in line with the previous study on elephant genetic diversity [23]. The low genetic diversity with the African savannah elephant may reflect a founder effect and highlights a serious problem for this endangered species.

A neighbor-joining tree (Supplementary Fig. 1) was constructed from the genetic distance data (Supplementary Table 3). Using the 402, 365, and 188 bp sequences, type 1 was grouped with *E. maximus*; types 2 to 5 were grouped with *L. cyclotis* (AY357274) and types 6 to 9 were grouped with *L. africana* (D84151 and the reference sample). The sequences of 365 and 188 bp fragments produced lower bootstrap values which showed lower support than using the 402 bp (data not shown). The genetic distance data and cluster analysis support the premise that not less than the 402 bp fragment should be used in phylogenetic studies for these species. But for forensic purpose, the three fragments exhibit sufficient information to identify the species of elephant from ivory samples and even the 188 bp fragment grouped samples to the correct species.

The strategy of nested PCR amplification and the sequence analysis method established by this study can be used to amplify highly degraded DNA from ivory and identify the species from which illegal imported ivory was obtained. The success rate of ivory identification was 84.3%. Based upon this study, about 97.9% of confiscated ivory samples originated from African elephant species; particularly the African savannah elephant (94.2%). It is anticipated that this test will assist in gaining information on ivory of previously unknown species.

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