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Case report

The identification of elephant ivory evidences of illegal trade with mitochondrial cytochrome b gene and hypervariable D-loop region

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ABSTRACT

DNA analysis of elephant ivory of illegal trade was handled in this work. The speciation and geographical origin of nine specimens of elephant ivory were requested by the police. Without national authorization, the suspect had purchased processed ivory seals from January to May, 2011 by Internet transactions from a site in a neighboring country. The DNA of decalcified ivory evidences was isolated with QIAGEN Micro Kit. The total 844–904 base pair sized sequences of mitochondrial cytochrome b and D-loop region could be acquired using direct sequencing analysis. They were compared with the sequences registered in GenBank. It was confirmed that most specimens were likely from African forest elephants (*Loxodonta cyclotis*), one from African savanna elephant (*Loxodonta africana*) and one from Asian elephant (*Elephas maximus*). Analysis of the mitochondrial hypervariable D-loop region sequence of elephants verified that one African savanna elephant might be from South Africa and one Asian elephant from Laos. Cytochrome b and D-loop region located in the mitochondrial DNA resulted in the successful determination of elephant DNA from nine processed ivory specimens.

1. Introduction

The ivory trade is the main reason for the decline in numbers of elephants such as African elephants (*Loxodonta cyclotis* and *Loxodonta africana*) and Asian elephant (*Elephas maximus*). The species identification of the ivory evidences is required for the enforcement of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulations. Beginning in 1993 year, CITES regulations have come into effect in South Korea. Some Asian communities carve their personal seal on the end of ivory cylinders to be used as a prestigious stamp.[1,2] These ivory products are derived from the core of the tusk and require a decalcification step before their DNA extraction.

Several conserved primers developed in mitochondrial DNA (mtDNA) regions such as cytochrome b,[3] 12S ribosomal RNA (rRNA),[4] 16S rRNA[5] and the hypervariable D-loop region[3,6] have been used for species identification and phylogenetic studies. This genetic marker analysis provides sensitive and useful tools for prevention of illegal hunting, poaching, and trade. The short-sized fragments of partial cytochrome b aid in species identification of highly processed or degraded forensic evidence.[7] The hypervariable D-loop region of mtDNA is particularly suitable for the genetic analysis of populations and closely related taxa.[8,9]

In the present case, police arrested an Internet-based ivory distributor, who had sold ivory products from January to May, 2011. The felon had purchased the ivory products from online shop in a neighboring country without any national permission. The seized ivory specimens against CITES regulations were tested to identify their species and geographical origins using partial cytochrome b and D-loop region.

2. Materials and methods

2.1. Specimens

Photographs of the nine ivory items seized by police are shown at Fig. 1. The ivory discs of about 50 mm in height were cut off with a dental drill and these discs were treated with 10–20 ml of 0.5 M EDTA for ten days. Chopped ivory (330 mg–500 mg) was treated with 1 ml of ATL and 100 μl of protease K in a QIAamp DNA Micro kit (QIAGEN, Valencia, CA, USA). DNA was extracted from 500 μl of the lysate. The elution volume was 80 μl. Amount and purity of extracted DNA was quantitated using a NanoDrop 2000 UV/vis

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Fig. 1. The ivory evidence. The item number (1–6) is inscribed under evidence. The lengths of the bottom plate of item 1 and item 2 are 3.3 cm × 1.35 cm. Items 3 and 4 have hexahedral shapes and their bottom length is 1.55 cm × 1.55 cm and 1.7 cm × 1.7 cm, respectively. Items 5 and 6 are round cylinders. The diameters of the two item 5 specimens are 1.1 cm and those of item 6 are 1.5 cm and 1.2 cm. The height of all items is about 5–7.5 cm.

spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The concentration for each evidence was as follows: item 1 (6.4 ng/μl), item 2 (4.8 ng/μl), item 3 (1.6 ng/μl), item 4-1 (6.8 ng/μl), item 4-2 (9.2 ng/μl), item 5-1 (11.3 ng/μl), item 5-2 (7.4 ng/μl), item 6-1 (3.1 ng/μl), item 6-2 (6.7 ng/μl). The average ratio of A260 to A280 was 1.24. There was not any positive control which available for an internal laboratory control for these ivory items.

2.2. Molecular markers and PCR amplification

The partial cytochrome b gene from 14850 to 15149 (numbered according to the human mtDNA sequence) was amplified by cyb-F and cytb-R (Table 1).[3,7] A segment of mtDNA including C terminal region of cytochrome b, the next threonine and proline tRNAs, and the rest noncoding control region of D-loop was amplified by MDL3 and MDL5[8] in order to discover the geographical origin of items. In case of samples could not be amplified with MDL3 and MDL5, primers AFDL1 ~ AFDL4 were used for their amplification.[9] Both PCR amplifications were performed in a 50 μl reaction mixture, which contained 16–92 ng of extracted DNA, 20 μM of each of primers, 2.5 unit of AmpliTaq DNA polymerase and reaction buffer of Gold ST★R 10X buffer (PROMEGA, Madison, WI, USA). Amplification was conducted in a 9700 thermal cycler (Applied Bio-Systems, Foster city, CA, USA) with 40 cycles of each amplification reaction.[3,7–9] All amplification reactants included negative controls that no DNA was added. PCR products were checked on a 2.5% agarose gel and purified with a QIAquick PCR purification Kit (QIAGEN).

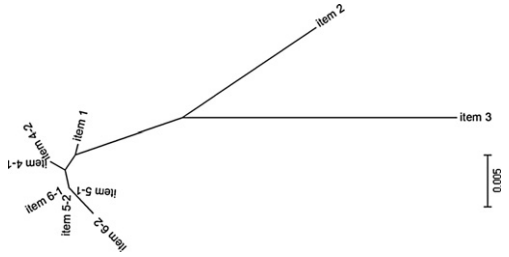

Fig. 2. The radial phylogenetic tree based on cytochrome b gene of nine ivory evidences by neighbor-joining method.

2.3. Sequencing analysis and BLAST search

The purified PCR products were sequenced directly using the aforementioned primers with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems). The unincorporated

Table 1
Primer sequences used in this case study.

Gene	Primer name	Primer sequence	Fragment length	Ta	Ref
Cytb	Cytb-F	5′-CCA TCC AAC ATC TCA GCA TGA TGA Aa-3′	357 bp	50 °C	3,7
	Cytb-R	5′-CCC TCA GAA TGA TAT TTG TCC TCA-3′			
D-loop	MDL3	5′-CCC ACA ATT AAT GGG CCC GGA GCG-3′	630 bp	63 °C	8
	MDL5	5′-TTA CAT GAA TTG GCA GCC AAC CAG-3′			
	AFDL1	5′-TTA CAC CAT TAT CGG CCA AAT AG-3′	400 bp	55 °C	9
	AFDL2	5′-TGA CAC ATT GAT TAA ACA GTA CTT GC-3′			
	AFDL3	5′-CTT CTT AAA CTA TTC CCT GCA AGC-3′	377 bp	58 °C	
	AFDL4	5′-GTT GAT GGT TTC TCG GAG GTA G-3′			

Ta: annealing temperature; Ref: reference.

Table 2
The polymorphic sites for the 357 bp fragment of the cytochrome b gene from nine specimens.

Specimens	0 2 7	0 6 3	0 7 6	0 9 3	0 9 6	1 0 2	1 3 8	1 4 7	1 5 0	1 9 5	2 3 1	2 4 6	2 6 1	2 6 7	2 7 6	2 9 7	3 0 6	3 3 3
Item 1	C	G	C	T	T	C	C	T	A	T	G	A	G	C	C	C	C	G
Item 2	T	A	T	.	.	.	T	.	G	C	A	G	.	T	.	.	.	.
Item 3	–	A	T	.	C	T	.	C	C	.	A	.	A	.	A	T	.	A
Item 4-1	T	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.
Item 4-2	T	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.
Item 5-1	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Item 5-2	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Item 6-1	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Item 6-2	T	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.

A period denotes a matching base with the top-most sequence. "–" means the site failed to obtain.