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# Identification of Substitute Ivory Product in Illegal Wildlife Trade using Morphological and DNA Based Analysis in Wildlife Forensics in India

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**Abstract** - The illegal trade of wildlife articles in the form of ivory, bones, horns, skin, hair and their derivatives has cost the lives and existence of many threatened species around the world. The increase in the price of ivory, because of short supply and high demand, as a result of ban on sale and display of ivory and its products in India have forced the craftsmen to use ivory substitutes (bones, resins, antlers, plastics and wood) which resembles the ivory to a much extent. Thirty two such suspected ivory plates having the paintings of Moghul era on them suspected to be made up of ivory material were seized by the law enforcement authorities at Delhi and were sent to the Wildlife Institute of India, Dehradun, India for the identification of species. We successfully identified the species using morphological and DNA based wildlife forensic techniques. The absence of both the fluorescence in response to ultraviolet (UV)-rays and Schreger lines on cross sections proved the non ivory origin of all three seized articles. The mitochondrial cyt b gene was sequenced for the identification of species of the seized suspected ivory products. Two suspected ivory plates which were randomly chosen from the seizure were found to be of water buffalo (*Bubalus bubalis*) on the basis of BLAST search at NCBI, USA and Neighbor-Joining (NJ) tree with high bootstrap values. Our results support the earlier reports of using substitute ivory and prove that these type of items still floats in illegal wildlife trade markets. Therefore, the development of DNA data base of all species threatened by the wildlife trade, on priority basis, will be very useful in identification of species of origin of seized articles and the implementation of wildlife protection laws and ultimately for the conservation of wild and other species.

**Key words:** *Substitute ivory, morphology, DNA, illegal wildlife trade, India*

### I, Introduction

The illegal trade of wildlife articles in the form of ivory, bone, horn, skin, hair and their derivatives has cost the lives and existence of many threatened species around the world. The concern about the seizure of such articles in the present continue to haunt the governments, conservationists and scientists who have put in their all efforts to save whatever wildlife is left on the Planet. In India the illegal wildlife trade was the biggest market for the manufacturers and buyers of ivory for many

hundreds of years till 1990 [1]. The ban on sale and display of ivory and its products in early 1990s (TRAFFIC, 1992) reduced this market to almost disappearance of this trade in India and shifted it to the South East Asian countries like Myanmar, Thailand, Cambodia, Laos, Vietnam and Singapore. The increase in the price of ivory, as a result of short supply and high demand, has forced the craftsmen to use ivory substitutes (bones, resins, antlers, plastics and wood) which too resembles to the ivory to distinguish them from the real [2]. The identification of finished products made from such ivory substitutes has been a great challenge in wildlife forensics. From India the Hindu religious idols and Moghul-style paintings on ivory plates are the most common products exported to other countries [2]. The morphological techniques i.e. the use of ultraviolet rays and presence of Schreger lines have been used widely to detect the ivory and substitute ivory products in the past [3 and 4]. Wildlife forensic genetics has employed the use of molecular genetic analysis in identification of species from a wide range of forensic samples in different studies around the world [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16]. However, the records of identification of fake wildlife articles specifically the fake ivory products up to the species level are hardly available. It is evident that isolation of DNA from any forensic sample is not an easy work, but when it has to be taken out from finished products it really becomes a challenging work. In this paper we describe the identification of species from such seized wildlife articles which later proved to be ivory substitutes using morphological and forensic DNA analysis methods.

## II. Materials and methods

**Case history:** Thirty two oval plates having the paintings of Moghul era on one side of them which were suspected to be made up of ivory material were seized by the law enforcement authorities in Delhi in 2008 (Fig. 1). These seized suspected ivory plates were sent to Wildlife Institute of India (WII), Dehradun, India for the identification of species they were made of.

**Samples for analysis:** Two suspected ivory plates which were chosen randomly out of 32 were used for species identification using morphological and forensic DNA based techniques.

**Morphological analysis:** Two morphological analyses were carried out to know if the seized articles were made of ivory material or not. The plates were put on UV-rays to give fluorescence because an ivory gives fluorescence when UV-rays fall on them [3]. Second test we performed was for the presence of Schreger lines, which are present in real ivory, on the cross sections of the plates [3 and 4]. The sides of the faces of the plates, which we estimated to be the cross sections, were searched for the presence Schreger lines.

### DNA based analysis for the identification of species:

*Isolation of the genomic DNA-* The genomic DNA isolation was done using small chips (500 mg) which were scraped from the opposite side of the painting on each plate with the help of a sterilized scalpel on a sterilized petridish. This was done to avoid any damaged to the paintings on them and the shape of the box. DNA was isolated by silica binding method in the presence of high concentrations of guanidinium thiocyanate (5M) to avoid the co-extraction PCR inhibitors [17].

*Polymerase Chain Reaction (PCR) amplification of cyt b mitochondrial gene-* The partial fragments of cyt b gene with universal primers of mitochondrial DNA were amplified for species identification [18]. The PCR amplifications was carried out in 25 µl reaction volumes containing 1x- PCR Buffer; 25mM MgCl<sub>2</sub>; 10 mM dNTPs; 10µM of each primer; 2.5U Taq polymerase, and 1µl of DNA for the optimum amount of amplification on 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, US). Cycling conditions were initial denaturation at 94°C for 3 min followed by 35 cycles for 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C and 10 min at 72°C with final extension. Amplification products were visualized by electrophoresis (6 µl) on a 2% agarose gel containing ethidium bromide. Amplified PCR products were purified using QIAquick® PCR purification Kit (QIAGEN, Germany). Cycle sequencing of PCR products was performed for these purified PCR products with their respective primers following the suggested composition of master mixture and PCR conditions on

9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, US). After cycle sequencing the PCR products were purified using "BigDye terminator clean-up" method. After cleanup these products were then subjected to sequencing on 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, US).

*Analysis of sequences for species identification-* The sequences thus obtained from two suspected ivory plates were BLAST searched on National Center for Biotechnology Information (NCBI), USA for species identification. The sequences were aligned using ClustleW Multiple sequence alignment [19], implemented in Bioedite software [20], with reference sequences of cyt b available at Wildlife Institute of India [Chital (*Axis axis*), Barking deer (*Muntiacus muntjak*), Sambar (*Rusa unicolor*)] and downloaded from the NCBI [water buffalo (*Bubalus bubalis*), wild buffalo (*Bubalus arnee bubalis*), Cattle (*Bos taurus*), Cattle (*Bos indicus*)] for the species identification., The accession numbers of all DNA sequences used in this study for cyt b are given in Table 1. The cyt b gene sequences of seized suspected ivory plates could not be submitted to GenBank because of their small size (<200 bp). Interested readers may request us for these sequences. To show the phylogeny between aligned sequences for cyt b gene the Neighbor-Joining (NJ) tree was constructed using Kimura-2 parameters for nucleotide substitution with bootstrap values using 1000 replications on MEGA software [21].

### III. Results and discussion

#### *Morphological analysis*

The morphological analysis of seized suspected ivory plates did not result in any conclusion as neither the fluorescence was detected under UV-light nor the Schreger lines were found to present the examined articles, which was expected if these were made up of ivory materials. Hence, the DNA based analysis was done for the identification of species of plates.

#### *DNA based analysis*

The method developed by Rohland and Hofreiter (2007) for the isolation of ancient DNA from old bone and teeth was specifically chosen for the isolation of DNA from suspected ivory plates as they were looking very old.

The isolated DNA was visualized on 0.8 % agarose gel on UV-transilluminator and it was found that very high intensity DNA was obtained successfully from the two seized suspected ivory plates and the box (Fig. 2). The PCR products of 472 bp lengths were amplified and visualized on 2.0 % agarose gel on UV-transilluminator for two samples for cyt b gene (Fig. 3). While doing BLAST search on NCBI two seized suspected ivory plates matched with the water buffalo (*Bubalus bubalis*) (Fig. 4A and B). To further confirm the results we aligned the sequences of two plates with the reference data base, available at WII. The aligned sequences were trimmed to make the consensus sequences of 198 bp. The Neighbour-Joining (NJ) tree made from sequences of cyt b using MEGA also showed the close proximity of these two seized suspected ivory plates towards water buffalo (*Bubalus bubalis*) with high bootstrap values (96%) (Fig. 5). The plates when seized were suspected to be made up of ivory materials which from our analysis came out to be made up of substitute ivory and probably from bone material on the basis of porosity, hardness and colour (white) of the samples. The care has to be taken while taking out the samples for the isolation of DNA from such type of articles to shirk any claims of distortion in their physical appearances, if proved to be from a legally permitted species. Even if the seized suspected wildlife products were came out to be fake and of substitute ivory, the entire process become a case of forgery which is an illegal practice. Thus morphological and DNA based techniques in the field of wildlife forensic not only help in identification of fake ivory products but in combating the forgery cases also. These results further supports the earlier reports of using substitute ivory [22] and showed that these type of items still floats in illegal wildlife trade markets. However, such articles poses problems in their identification on the basis of morphological analysis when they are not present in their complete structure, as evident from this study and imitations of rhino horn, elephant ivory, claw sand skin of tiger and

leopard and animal pods. The development of DNA data base of all species threatened by the wildlife trade, on priority basis, will be very useful in identification of species of origin of seized articles and the implementation of wildlife protection laws and ultimately for the conservation of wild and other species.

#### IV. Acknowledgments

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Table 1. Accession numbers of the sequences of the Texa used in this study.

S. No.	Common name (Species)	Gene bank accession no.	Reference
1	Suspected ivory plate 1	Available on request	This study
2	Suspected ivory plate 2	Available on request	This study
3	Water buffalo ( <i>Bubalus bubalis</i> )	EU908278.1	Sahajpal <i>et al</i> (Unpublished)
4	Wild buffalo ( <i>Bubalus arnee bubalis</i> )	D32193.1	[23]
5	Cattle ( <i>Bos taurus</i> )	D34635.1	[24]
6	Cattle ( <i>Bos indicus</i> )	AY689190.1	[25]
7	Barking deer ( <i>Muntiacus muntjak</i> )	Available on request	This study
9	Sambar ( <i>Rusa unicolor</i> )	Available on request	This study
10	Chital ( <i>Axis axis</i> )	Available on request	This study



Figure 1. The seized suspected ivory plates having paintings.

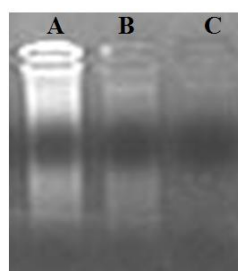


Figure 2. Images of genomic DNA. Legends; lane A and B= suspected ivory plate 1 and 2, respectively, lane C= negative control.

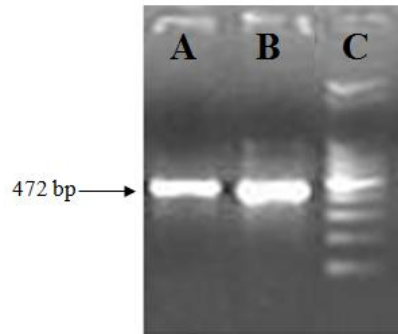
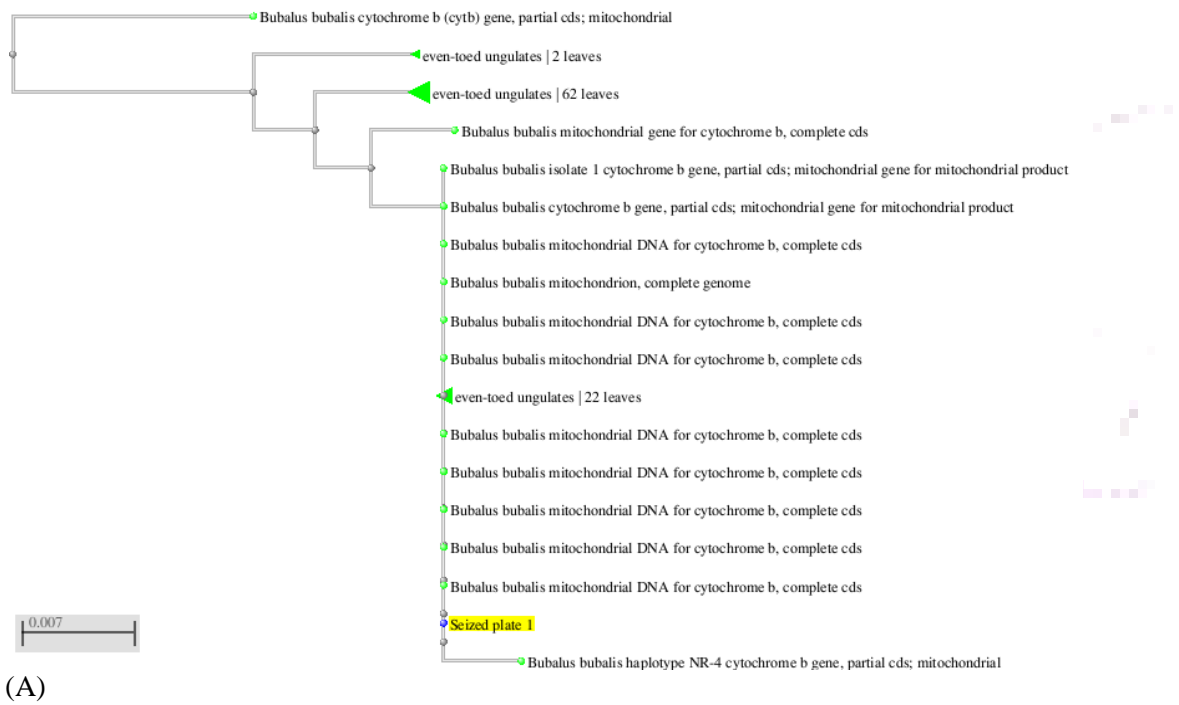


Figure 3. PCR amplified products for partial fragment of cyt b gene. Legends, lane A and B = suspected ivory plate 1 and 2, respectively, lane C= DNA ladder of 100 bp



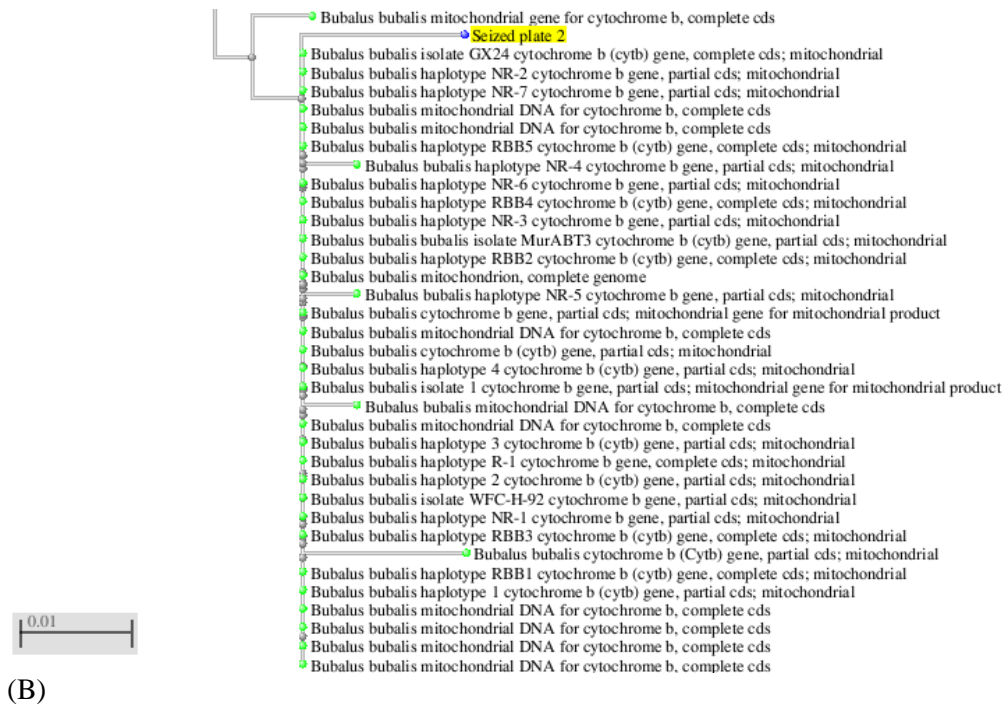


Figure 4. Dendrogram made from the BLAST pairwise alignments of cyt b sequences of two suspected ivory plates (A and B).

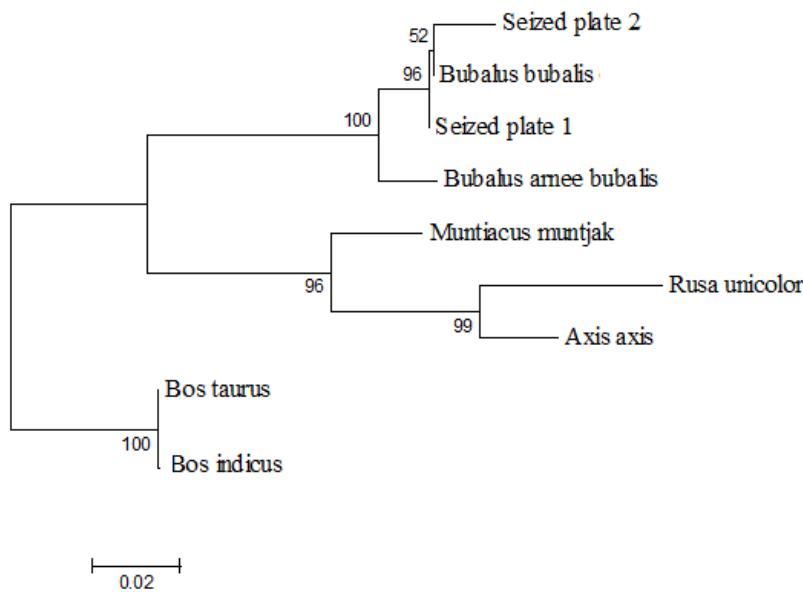


Figure 5. The Neighbour-joining tree made from cyt b sequences. The numerical values at the nodes represent the bootstrap values above 50% with 1000 replicates.

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