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Multilocus Genotyping of Asian Elephant Ivory: A Case Study in Suspected Wildlife Crime

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Abstract

As a part of wildlife crime investigation, two pieces of Asian elephant (*Elephas maximus*) ivory were collected by Uttarakhand forest department at different time interval to identify whether they belonged to same individual. The samples were analyzed using multilocus genotype based genetic analysis. Twelve fluorescent labeled microsatellite loci were used to analyze the nuclear DNA that were developed and validated for Asian elephants. Based on DNA typing, both the samples showed same alleles at every tested locus indicating that both the ivory pieces originated from single individual. The study highlights successful extraction and analysis of nuclear DNA employing microsatellite markers from ivory collected from Asian elephant.

Keywords: Wildlife crime investigation; Asian elephant; Nuclear DNA; Ivory; Microsatellite loci; Multilocus genotype

Introduction

Asian elephant (*Elephas maximus*) is one of the charismatic and endangered species in the world. Male Asian elephant posses well developed tusk and have been exploited for ivory. Poaching for ivory is major declining factor for the Asian elephant population and causing the imbalance male female ratio in the wild [1]. The trade of elephant ivory is restricted by Convention on Illegal Trade in Endangered Species (CITES) and Wildlife (Protection) Act, 1972 of India. Hence there is a grave need to confiscate and identify the parts and products made up of elephant ivory.

Schreger lines are the major identifying features of elephant and Mammoth ivory, which can be observed in a cross section of the same [2]. Identifying processed ivory and ivory object becomes more challenging since Schreger lines may not be visible in the processed ivory. In support of this, DNA marker technologies have revolutionized the evolutionary and conservation genetics over the last couple of decades. It has successfully contributed for the strengthening of wildlife enforcement and management of threatened species for the conservation of biodiversity [1,3,4]. Extraction and analysis of DNA from ivory is a relatively challenging task, however in recent past; few studies have demonstrated the analysis of DNA from elephant ivory [5-9]. Hence, there is a grave need to increase the use of genetic approaches for wildlife enforcement and management.

One complete tusk of Asian elephant found in a forest area of Uttarakhand (a state of North India), and was stored by forest official. Subsequently, one suspicious carcass of male elephant with one missing tusk was found in the same forest area. In this situation, it becomes essential to know whether the cause of the death of the animal was poaching. Therefore, small pieces (approximately 2 square inches) of the ivory from both the tusk including earlier stored and the one from carcass of the elephant was forwarded to identify whether both ivory pieces belong to the same individual.

Microsatellites are the best available markers for individual matching and also for identifying parents, offspring and close relatives in captive and wild population [3,10]. Present study highlights about the successful extraction of DNA from small pieces of Asian elephant ivory and analysis of nuclear DNA by using microsatellite loci. These microsatellite loci were developed for Asian elephant population in Thailand [11]. Furthermore, we have tested these markers on more than ten different Asian elephants of India and found them to be highly polymorphic in tested elephants. These markers are having great potential for use in identifying individuals and their relatives in wild and captive population of Asian elephant and are favorably used in our lab for dealing such cases on routine basis.

Material and Methods

Extraction of DNA

Approximately 2 cm^2 pieces from both the ivory sample were pulverized separately to granular form by using stainless steel homogenizer. The granulated ivories were incubated separately with 0.5 M EDTA (pH 8.0) for 72 hours for decalcification with regular rotation at room temperature in a hybridization oven. Old EDTA solution was replaced with fresh solution on every 8-10 hrs. Commercially available DNeasy Tissue Kit (QIAGEN, Germany) was used to extract DNA in final extraction volume of 40 µl from both the decalcified ivory granule according to the manufacturer's protocol.

PCR amplification and electrophoresis

Both the DNA samples along with one positive control DNA of a known Asian elephant and one negative control were subjected to PCR amplification by using 12 Asian elephant specific microsatellite loci (Table 1) [11]. PCR amplifications were performed in GeneAmp PCR System 2700, (Applied Biosystems, Singapore) in a final volume of 20 µl, containing 2 µl of extracted DNA, 1× PCR buffer (Applied Biosystem), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 3 pmole of each primer and 0.5 units of AmpliTaq Gold DNA polymerase (Applied

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Biosystem). Amplification condition was 95°C for 10 min followed by 35 cycles at 95°C for 45s, 58°C for 45s and 72°C for 1 min, with a final extension of 72°C for 10 min. The PCR reaction was repeated three times and all the PCR products were genotyped using ABI 3130 genetic analyzer (Applied Biosystem).

Validation of microsatellite marker on Indian elephant

DNA extracted from the biological samples of 10 Indian elephant individuals were used for validation of all the 12 microsatellite markers to explore the polymorphism pattern on Indian population which were developed for Asian elephant of Thailand [11]. The same set of markers is used for dealing the similar cases on routine basis for Indian elephants.

Analysis of genetic data

Allele sizing was done with the help of GeneMapper software (Applied Biosystem). The multilocus genotype profiles of both the ivory pieces were subjected to relatedness testing by calculating likelihood relatedness (LR) using *ML-relate* software [12]. The multilocous genotype of other Asian elephant of Uttarakhand, India was also used to compare the LR.

Primer name	Primer Sequences (5′-3′)
EMU03	F: AGAAGCAAAACCCATGAAGC
	R: TTGAAACTTGCCAGCCTCTT
EMU04	F: TGACTCTCCCTCTTCTGCATC
	R: GGCTGAGAGGGAAAGAAATTG
EMU06	F: TTTTTGGGGCTAGAAACTGG
	R: CCCAGTGTTCAATAGATGCTTT
EMU07	F: GAGCAGTGCCTTTCGTGAC
	R: AGCCTGGGAGGTAAGTAGCA
EMU09	F: TCCGTAATTGCACACTTTTAGC
	R: ATGAGGGGTAATGAGGGTCA
EMU10	F: AATCGACTCAGCAGCAACAG
	R: CCAGTAAATCCATATCACTCGTC
EMU11	F: CAATATGGGTGTGGGTTTCC
	R: GAAATGCAGCATAAATAATATCATGG
EMU12	F: CCAAAGAAGACCCATGTTCC
	R: CTGACTATGGGGGAGACTGC
EMU13	F: GTATTTGGGCTGGCATGGT
	R: GTGGGGTCTGTGGTCAAGTG
EMU14	F: GCCTACATGCAGGGTTTGC
	R: TGAGCCTCTGGCATTTATGA
EMU15	F: TTCGGGATGTTCTCTTCTGT
	R: GGGGCTTAACTAATAGGCTTCA
EMU17	F: CACTCAGAGTTCCAAGAAGCAG
	R: TGCCAGCCATTTCCTCTC

Table 1: List of primer sequences used for genotyping of ivory DNA [11].

Locus	Ivory-I		Ivory-II	
	Allele I	Allele II	Allele I	Allele II
EMU03	134	140	134	140
EMU04	104	116	104	116
EMU06	149	149	149	149
EMU07	102	114	102	114
EMU09	160	160	160	160
EMU10	094	102	094	102
EMU11	123	123	123	123
EMU12	137	137	137	137
EMU13	107	107	107	107
EMU14	133	133	133	133
EMU15	154	154	154	154
EMU17	123	137	123	137

Table 2: Observed allele size in base pair (bp) in Ivory-I and Ivory-II.

Results and Discussion

DNA extracted from both the ivory was of good quality and all the 12 microsatellite loci were amplified successfully in all the three repeats. Different alleles were observed at most of the loci in all known unrelated elephant individuals. The multilocus genotype obtained from both the ivory pieces indicates that both the ivory were having the same allele at each locus (Table 2). The multilocus genotype of both the ivory sample is 134140, 104116, 149149, 102114, 160160, 094102, 123123, 137137, 107107, 133133, 154154 and 123137. The relationship calculated between two ivory pieces by estimation of LR value was 1.00, which indicates that both the sample belong to biologically same individual. The LR value between ivory and other tested Asian elephant was 0.00, which testifies the significance of the microsatellite loci used in this study for addressing same situation for Indian elephant population.

Our analysis indicates that both the tusk of this animal were recovered, therefore; the cause of death of the animal was not poaching. This study is an elite example of the use of scientific protocol for proper wildlife management. Extraction and analyses of DNA from ivory of African elephants were first time demonstrated by Comstock and associated colleagues [5], which helped in development of DNA extraction protocol for Asian elephant [6-7,9]. These protocols indicated that they can be deployed for wildlife enforcement by detection of ivory and ivory products derived from elephants. Genetic characterization of the ivory of African elephant established the assessment of the place of its geographical origin by using microsatellite markers [8]. Recently, mitochondrial DNA marker was successfully amplified using template DNA extracted from ivory object in a forensic case [4]. Present study highlights the successful analysis of nuclear DNA from ivory pieces of Asian elephant and represents a worthy example for use of scientific approach for proper wildlife enforcement and management.

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