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Resolving the *trans*-boundary dispute of elephant poaching between India and Nepal

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ABSTRACT

In Kangchenjunga Landscape (KL), which is shared by three countries – Bhutan, India, and Nepal, the wild elephants migrate from east of Jhapa (Nepal), through West Bengal (India) and Sibsoo (Bhutan) to further east in Assam (India). The route Jhapa-WB-Sibsoo-Assam is a known route for elephant movements where maximum causalities have been reported in the past. The present study was undertaken to ascertain the individual identity of a poached elephant in Jhapa, Nepal and ivory which was suspected to be from the same individual elephant confiscated in Siliguri, India. We undertook STR profiling of the confiscated specimens with nine polymorphic STRs. The forensic parameters has established the fact that the two analyzed samples of elephant were not identical and belong to two different individuals. The present study highlights the necessity of transboundary research for elephant conservation and monitoring their movements in Kangchenjunga Landscape and emphasizes the use of forensic genetics in curbing illegal wildlife trade.

1. Introduction

The fate of humans and elephants have been knotted through centuries in the Indian subcontinent and this particular lyevokes a strong emotional reaction in India. The elephant is an integral part of the forest ecosystems, which in turn provide ecological services essential to human survival. Sadly, despite being acclaimed as man's best friend, the elephant is now in frantic straits in the wild due to poaching and loss of habitat. Throughout their distribution range in India, the elephant is increasingly threatened and affected by habitat loss and fragmentation, human-elephant conflict, and poaching for ivory [1–3]. Among different conservation threats, poaching for ivory is the major threat for elephant conservation in Asia. Tusk develops only in the male Asian elephant and has led to the skewed killing of Tusker [4]. However, there are other type of males without tusks, also known as Makhna, often sub-ordinate to the tusker in the Asian Elephant. This phenomenon is absent in the African elephant. Legal and illegal hunting has taken a great toll on India's elephant populations over the past two centuries [5,6]. As reported by the Wildlife Protection Society of India (WPSI), almost 371 elephants have been poached in India between 2006 and 2017. Though other agencies claim there has been poaching of over 2000 male elephants in south India alone (http://www.dalitstan.org/tamil/stfreprt.html). These have resulted in the loss of 95% of their historic range and only 24000–28500 elephants are roaming in different regions of India [1].

Further, the movements of elephants cannot be bound by the political boundaries, especially in the absence of geographical barriers. The Kangchenjunga Landscape (KL), which is shared by three countries – Bhutan, India, and Nepal, was once home to large herds of wild elephants that migrated from east of Jhapa (Nepal), through West Bengal (India) and Bhutan to further east in Assam (India) [7]. Much of the elephant habitat in KL has been altered significantly through land conversion into settlements, agricultural land, tea gardens, or teak plantations, which has affected the foraging behavior and their migration paths. As a result, elephants often enter settlements and cultivated lands destroying both infrastructures, as well as crops, and occasionally causing human

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fatalities. Retaliatory measures, such as culling and electric fences, result in death or injury of elephants. The trade of elephant ivory is restricted by the Convention on Illegal Trade in Endangered Species (CITES), Wildlife (Protection) Act, 1972 of India, National Parks and Wildlife Conservation Act, 2029 (1973) and CITES Act, 2073 (2017) of Nepal. Identification of species using genetic and morphological tools is crucial for the different law enforcement agencies. Furthermore, identifying individuals in wildlife forensic cases is crucial in certain issues such as poaching, where it may be necessary to demonstrate that a horn, tusk, bone or skin has originated from a specific individual or identifying a number of individuals poached in a seizure. DNA profiling techniques can provide key evidence to wildlife crime investigations [8].

The advancement in DNA forensics tools in recent years facilitate species identification from varied biological samples [9–13], sex identification [14,15], paternity assessment and individual identifications [16] and establishment of the origin of a particular individual [17] from hair, feces, saliva, bloodstains, urine, etc. Wildlife forensic genetics have been used in dealing with poaching cases [3,12] troubles involving particular individuals in conflicts has been illustrated by the establishment of the genetic identity of a serial killing wolf [18], bear-human conflict [14,15,19], tiger-human conflict [20]; and crop-raiding elephants [21]. Thus, in the present study, we used a forensic genetic approach to establish the individual identity of confiscated materials received from India and Nepal. This study provides an example of the feasibility of molecular tools in developing effective management strategies to deal with the wildlife poaching cases in KL.

2. Case history

In this study, we received two confiscated samples of elephant as wildlife offenses under the transboundary crime between India and Nepal due to the suspected link of poachers and middle men, residing in Indian and Nepal. We received six pieces of elephant ivory weight ranging from 0.50 to 3.4 kg from the Office of the Directorate of Revenue Intelligence (DRI), Siliguri Regional Unit, Darjeeling, West Bengal, India. The seized ivory was recovered from the possession of two persons who had smuggled the same from Nepal to India. It was observed that tusks appeared to be relatively fresh and extracted recently. Further, intelligence collected from Nepal had found a recent incidence of elephant killing in Nepal at Budhbare area which is close to the Indo-Nepal border (Fig. 1). Concerns were raised from the law enforcement agency that ivory recovered and seized by them might have been cut off from the elephant found dead in Nepal. ZSI also received body remains (piece of ivory ~12.3 gm and 50 gm flesh) from the Office of the Dept. of National Parks & Wildlife Conservation (DNPWC), MoEF, Govt. of Nepal to find out whether it matches with the DNA of the seized ivories in India.

3. Materials and methods

3.1. DNA extraction and species identification

Genomic DNA was extracted from tissue remains of pith and blood stains from the ivory received from Siliguri, India and the muscles/flesh from the seizure (#1 sample) received from Nepal following Qiagen DNeasy Blood and Tissue kit (Qiagen, Germany). All DNA extraction procedures were done in aseptic conditions. We sequenced the partial fragment of Cytb gene using universal primers following standard PCR protocol [22] from the flesh sample received from Nepal. Amplified PCR products were subjected to ABI 3730 Genetic Analyzer after Exo-SAP treatment and Big dye cycle sequencing PCR for sequencing. The quality of the DNA sequences was determined using Sequence Analysis software (Applied

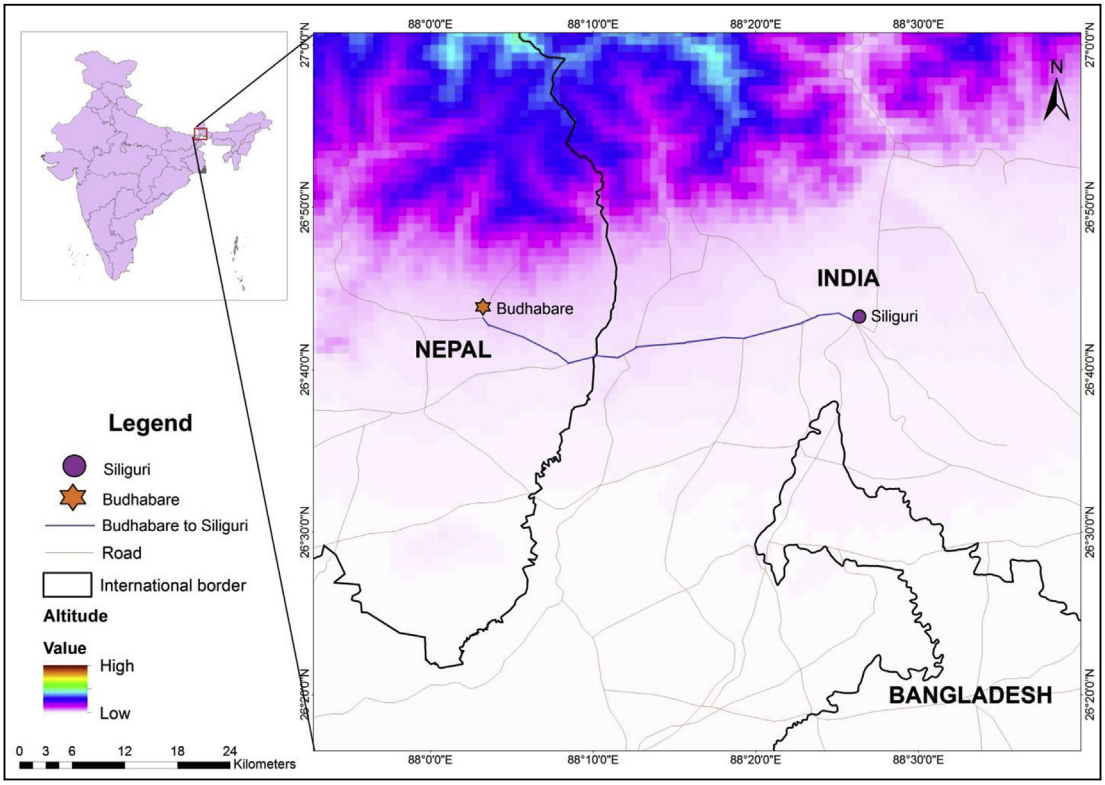

Fig. 1. Map showing location of Ivory confiscation in Siliguri, India and location in Budhabare, Jhapa, Nepal where a dead elephant was reported.

Biosystems) and edited by Sequencher 4.7 (www.genecode.com). Cleaned sequences were BLAST at NCBI and on basis percent similarity species were identified.

3.2. Individual identification and assessment of forensic parameters

We used nine polymorphic di-nucleotide microsatellite loci i.e. EMU4, EMU7, EMU9, EMU 10, EMU 11, EMU 12, EMU 13 EMU 15 and EMU 17 specifically isolated for Asian Elephant and characterized on Thailand elephant population by Ref. [23]. [23] reported these loci were polymorphic in Asian elephant and observed 3 to10 alleles. These STRs have been used for the applications of individual identification including forensic analysis of Asian elephant in India [3] and Chakraborty et al., 2014). PCR amplifications of all the nine markers were carried out in a ABI Veriti™ Thermal Cycler (Applied Biosystems, USA). Each PCR reaction volume of 10 μl contained 2ul of the DNA, 5 μl of the 1 × multiplex PCR Master Mix buffer (QIAGEN Multiplex PCR Kit, Germany), 1 × BSA and 0.2 μM of each primer. The thermal profile for amplification of the microsatellite loci was initial denaturation at 94 °C for 15 minutes, followed by 40 cycles of denaturation at 94 °C for 35 seconds, annealing at 53 °C for 1 minute and extension at 72 °C for 90 seconds, with a final extension for 30 minutes at 72 °C. One positive (DNA sample of a known elephant) and one blank sample were included in each PCR amplification reaction. The amplified PCR products were subjected to fragment analysis in an ABI 3730 Genetic Analyzer (Applied Biosystems, USA) using POP-7 polymer. The International Society for Forensic Genetics (ISFG) has recommended the use of the allelic ladder to validate the size of allele present in specific species during Individual identification. However, allelic ladders are unavailable for the wild species like an elephant. Therefore we followed guidelines suggested by Ref. [24] and standard PCR products were used to identify the likely shift in allele size. A similar approach has been used in a study on the Bengal tiger [20] and brown bear [19]. To avoid the genotyping error which can bias the individual identification [25], we typed all samples three times through independent PCR runs as a multi-tube approach is commonly recommended to minimize errors due to allele dropouts and false alleles. GeneMapper v4.1 (Applied Biosystems, USA) was used for the manual scoring of the allele. Allele quality was determined by the peak height, i.e. Relative fluorescent Unit (RFU) height between 1000 and 32000 for all the loci. After scoring and re-arrangement of multi-locus genotype data were processed further for understanding the allele composition in the analysed samples. We investigated forensic parameters such as number of alleles, polymorphism information content (PIC), probability of match (PM), power of discrimination (PD) and power of exclusion (PE) using the online software - STR analysis for forensics (STRAF 1.0.5) (Gouy and Zieger, 2017).

4. Results and discussion

4.1. DNA extraction and species identification

We processed all seven samples (six pieces of ivory and one flesh sample) for the genetic analysis but finally were able generate data from four samples (three ivory samples and One flesh). The NCBI blast search indicated 100% similarity for all the four confiscated materials with the Asian Elephant (*Elephas maximus*) sequence available on NCBI (GenBank accession no. AF540924) and published by Verma and Singh (2003). Therefore, we concluded that all the four samples provided by the authorities and examined by us were from the Asian Elephant (*Elephas maximus*).

Table- 1
Genetic profiling of Elephant (*Elephaus maximus*) samples.

Sample ID	EMU10		EMU11		EMU12		EMU13		EMU15		EMU17		EMU4		EMU7		EMU9	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
I_Tissue remains_India	94	94	124	124	139	141	101	105	140	140	121	127	97	103	102	114	160	160
II_Blood stains_India	94	94	124	124	139	141	101	105	140	140	121	127	97	103	102	114	160	160
II_Tissue remains_India	94	94	124	124	139	141	101	105	140	140	121	127	97	103	102	114	160	160
III_Tissue remains_Nepal	90	96	133	133	0	0	101	105	140	140	134	134	97	103	102	114	160	160
Control sample- Known Elephant	94	94	124	128	144	148	105	107	138	142	121	121	101	103	110	110	162	162

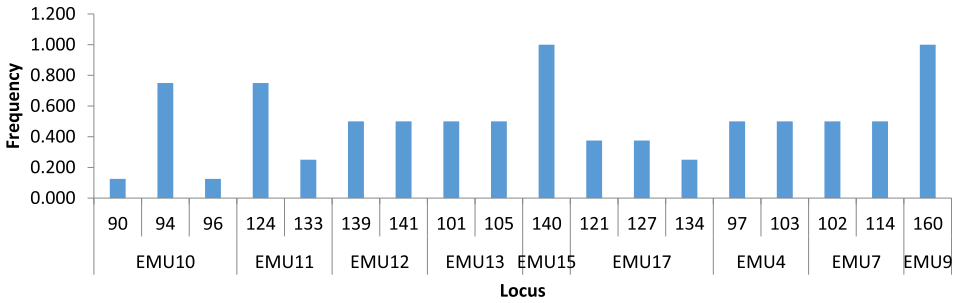

Fig. 2. Locus wise allele frequency in Elephant samples used in the present study.

Table- 2
Unique alleles to ascertain processed samples originate from different individuals.

Sample ID	EMU10		EMU11		EMU17	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
I_Tissue remains_India	94	94	124	124	121	127
II_Blood stains_India	94	94	124	124	121	127
II_Tissue remains_India	94	94	124	124	121	127
III_Tissue remains_Nepal	90	96	133	133	134	134

4.2. Genetic profiling, individual identification and forensic investigation

All genotypes were of high quality (RFU >1000, <32000). All the nine microsatellite markers except locus EMU-15 and EMU9 were polymorphic. Altogether, nine STRs yielded 18 alleles (Table 1) and it ranges from 1 (EMU9 and EMU15) – 3 (EMU10 and EMU17). Allele frequencies of all nine markers in the present study ranges from 0.13 to 1.0 (Fig. 2). Theses markers have been also used in different studies and allele diversity ranges from 3 to 10 in Asian elephant population in Thailand [23] and 3–5 in India (Chakraborty et al., 2014) Of the nine STRs, six loci, *i.e. EMU12, EMU13, EMU15, EMU4, EMU7 and EMU9* showed identical alleles in all the samples. However, three STRs, *i.e. EMU10, EMU11* and *EMU17* exhibited variable alleles between the samples received from India and Nepal (Table 2). Locus wise forensic parameters *i.e.* PIC, PM, PD, Hobs and PE are presented in Table 3. The combined power of discrimination (Comb PD) and power of exclusion (Comb PE) of the select panel of nine STRs was 0.875 and 1.0, respectively. Further, the observed combined probability of match (Comb PM) of the select panel was 0.125 which has been conclusive in establishing the fact that the two analyzed samples of elephant were not identical and belong to two different individuals. The probability of identity (PID), which is the probability of two individuals drawn at random from a population sharing the same genotype, is affected by the population size and the level of heterozygosity [26]. The present study indicated that the combination of eight to nine loci was adequate for individual identification with a PID 6.8×10^{-4}. We believe that this PID is acceptable for discriminating elephant individuals within the population sharing boundary with the India and Nepal. However, the census size of the elephant in West Bengal in still unknown, therefore it would not be possible to estimates exact PID and PID (sib). Other studies on the different populations of Asian elephant suggested that five or six loci are adequate for individual identification at the PID (3.18×10^{-8}) level [27]. Thus, we conclude that the use of nine microsatellites was adequate for individual identification to ascertain whether all the four samples were genetically identical. Based on the differential allelic patterns revealed by three STRs, *i.e. EMU10, EMU11* and *EMU17*, we conclude that all samples collected from India and Nepal were from the two different individuals.

5. Conclusion

This study has established that the two seizures as received at ZSI, Kolkata for matching DNA fingerprints from the body remains, one from India and other from Nepal, originated from two different individuals. The present study highlighted the need for the *trans*-boundary research between India, Nepal and Bhutan for elephant conservation. Loss of habitat in the landscape is detrimental to forest-dependent wildlife species due to the loss of prey or forage, or the disruption of habitat connectivity, particularly for migratory

Table 3
Genetic diversity indices and forensic parameters of Elephant samples.

Locus	Na	PIC	PM	PD	Hobs	PE	Comb PM	Comb PD	Comb PE
EMU10	3	0.555	0.500	0.500	0.500	0.188	0.125	0.875	1.000
EMU11	2	0.375	0.500	0.500	0.000	0.000			
EMU12	2	0.375	1.000	0.000	1.000	1.000			
EMU13	2	0.375	1.000	0.000	1.000	1.000			
EMU15	1	0.000	1.000	0.000	0.000	0.000			
EMU17	3	0.555	0.500	0.500	0.500	0.188			
EMU4	2	0.375	1.000	0.000	1.000	1.000			
EMU7	2	0.375	1.000	0.000	1.000	1.000			
EMU9	1	0.000	1.000	0.000	0.000	0.000			

Where, Na- Number of alleles, PIC- Polymorphic Information Content, PM- Probability of match, Power of discrimination, Hobs- Observed heterozygosity, PE- Power of exclusion, Comb PM- Combined probability of match, Comb PD- Combined Power of discrimination, Comb PE- Combined power of exclusion.

wildlife species such as elephant, tiger and snow leopard. In the lowlands of KL, elephants are known to migrate from Koshi Tappu Wildlife Reserve in Eastern Nepal through Darjeeling and Jalpaiguri, West Bengal, India, and Bhutan to Assam in North-East India. Understanding the behaviour and population dynamics of such transboundary migratory species is hence important for addressing human-wildlife conflicts and status of ivory trade in this landscape. Further, it is also important to estimate the population size, demographic history, gene flow between elephant populations in this landscape for better understanding and management. Hence the present study proposed to initiate a joint effort by India, Nepal and Bhutan to establish genetic data of elephant in this region to assign the source of origin of confiscated materials and understand the biology of an elephant.

Declaration of competing interest

Authors declare there is no conflict of interest.

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