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Genetic Based Species Identification and Tracking of the Geographic Origin of a Fully Tanned Animal Skin in Wildlife Forensics

Abstract

Species identification and assign the geographic origin from processed products based on morphological traits is a challenging task in wildlife forensics due to lack of the reference specimens. Here, we report species identification and assign the geographical origin of a fully tanned animal skin using complete mtDNA cytochrome b gene. A nucleotide sequence of 1140 bp cytochrome b was generated from the DNA extracted from the small piece of skin from the inner ear (IEP-01). GenBank BLAST search of the unknown Cyt b (1140bp) sequence against the full range of published *Rangifer tarandus* has facilitated in identification of species and ascertaining the species of origin with high meta probability (100%). We determined that the seized unknown skin is wild reindeer (*R.t. groenlandicus*) and has been originated from Canada, where this Least Concern species under the IUCN red list. We propose to establish genetic database across the range of the species threatened due to illegal trade to determine hotspots of poaching.

Keywords: Reindeer(*Rangifer tarandus*); Forensic genetics; Tanned skin; Species identification; Geographic origin; Cytochrome b gene

Case Report

Volume 2 Issue 3 - 2016

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Received: June 07, 2016 | **Published:** June 16, 2016

Introduction

Illegal trading of wild animals, their body parts and derived products is a major concern in wildlife conservation. Species identification based on DNA analysis is a critical tool in wildlife forensics, especially when the specific morphological characteristics are lost due to transformation or processing [1]. Mitochondrial markers, such as cytochrome b, 16S rRNA, 12S rRNA and Cytochrome Oxidase showing interspecific variation, are commonly used to perform species identification and phylogenetic studies in wildlife forensics [2-5]. Cyt b is highly informative in mammals and a large database for this genetic marker is already available for different species and subspecies in different geographical ranges, making it especially useful in wildlife forensics [6]. Assignment of samples, we need to amplify large fragment of mtDNA gene that show more number of SNPs over a geographic range. In most of the wildlife forensic cases, the samples received are in highly degraded condition or highly processed, viz., burn bones/bone piece, cooked meat, tanned skin, claws, canines, etc. Hence, the genomic DNA will be in low quantity and quality as well. Therefore, extraction of a good quality DNA from such type of degraded and tanned sample is a challenging task for the wildlife forensic experts.

In the present study, we aimed to select proper part to high yield DNA from highly tanned animal skin and amplify the large fragment of mitochondrial gene to identification of species and to ascertain its geographical origin that was seized by the Custom Authority at Indira Gandhi International Airport, New Delhi India.

Case History

A highly tanned animal skin was seized by the Custom Officer at Indira Gandhi International Airport, New Delhi, India, during December, 2012 and sent to the Wildlife Institute of India, Dehradun for species identification (Figure 1). The skin at visual inspection appeared to be of a deer species but the morphological hair patterns did not show definite species assignment due to the lack of an extensive collection of reference samples for comparison. A DNA based species identification strategy was devised to overcome this problem.

Materials and Methods

DNA extraction

Since the sample was highly tanned, we attempted DNA extraction with commercially available Qiagen DN easy Tissue Kit (QIAGEN, Germany) following manufacturer instructions. Three samples from different parts of unknown tanned skin (IEP-1, OSP-2 and OEP-3) were taken for DNA extraction. The tanning of the skin potentially caused DNA degradation, therefore a section of the inner part of the animal's ear (IEP-1) that seemed to be relatively less affected by the chemical process was selected for DNA extraction and we processed remaining samples i.e. the outer part of skin and ear (OSP-2 and OEP-3) in order to compare the efficiency of DNA extraction from the different parts of the tanned skin.

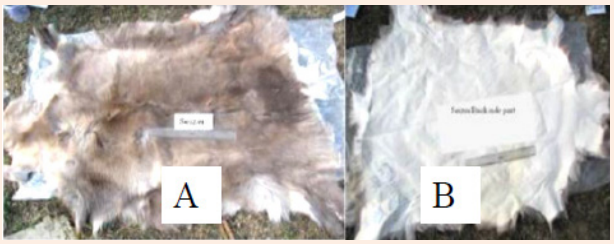

Figure 1: Animal skin seized by the customs authorities at Indira Gandhi International Airport, New Delhi, India. A, dorsal view; B, ventral view.

To determine the quality and concentration of DNA obtained, the samples were subjected to gel electrophoresis on a 0.8% agarose gel in 1X TAE buffer and DNA quantified with a UV spectrophotometer (Amersham Pharmacia) (Figure 2A).

PCR Amplification and Sequencing

DNA template were subjected to polymerase chain reaction using universal of Cyt b gene of different size 200bp, 350bp and 1140bp [7-9]. All PCR reactions were carried out on an Applied Biosystems® 2720 Thermal Cycler (ABI) in a total reaction volume of 25 µl containing 10 µl 2X PCR mix buffer (Amresco); 10 µM of each primer, and 4 µl of total DNA. The Thermal cycling consisted of conditions of denaturation step at 94°C for 3 min, 35 cycles of denaturation(94°C for 30 s),annealing (53°C for 45 s) and primer extension (72°C for 40 s) and a final extension step of 10 min for 72°C 10 min. A small volume of PCR products (5 µl) were subjected to electrophoresis on 2% agarose gel and visualized over an UV transilluminator. Extraction and PCR blanks were incorporated into the analysis. One extraction blank was incorporated with three samples extracted, and one PCR blank was subsequently incorporated with every three extracts that were amplified.

In this study, we used only (IEP-1) PCR amplicon for DNA sequencing. Amplified 1140 bp PCR product was purified using Exo-SAP to remove residual oligonucleotides and dNTPs prior to sequencing reaction. The forward and reverse primers were used independently for the sequencing reactions using the Big Dye® Terminator v3.1 Cycle Sequencing kit to generate sequence from both ends. The products were purified using a standard ethanol precipitation method and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Data analysis

Cyt b sequence (IEP-1) was cleaned and validated using SEQUENCHER 4.8 (Gene Codes Corporation, Ann Arbor, MI). Multiple sequence alignments were performed using the CLUSTAL W algorithm implemented in BIOEDIT version 7.0.5.3 \ 9 [10]. The sequence obtained from the unknown skin specimen was compared with the sequences publicly available at GenBank using BLAST search tool of NCBI (http://blast.ncbi.nlm.nih.gov/). All the sequences that showed similarity with the unknown sequence (IEP-1) were downloaded (Tables 1 & 2) and used for phylogenetic analysis using Kimura 2 parameter distance matrix with the neighbor-joining method as implemented in Mega v5.0 software [11].

Results and Discussion

Of the total samples (n = 3), DNA extracted from, two samples (OSP-2 and OEP-3) did not yield good DNA (Fig 2.A) and amplification was not observed after PCR (Figures 2A & 2B). Sample IEP-1 yielded detectable product of all three different size (200bp, 350bp and 1140bp) Cyt b gene on agarose gel electrophoresis (Figure 2B).

In case of non amplified samples (OSP-2 and OEP-3) we suspected the presence of PCR inhibitors within the DNA, or inhibitors may be due to chemical used in tanning process or from the environment. Therefore, we tested the DNA for PCR inhibitors, by using known positive PCR reactions. The results indicated that two DNA samples (OSP-2 and OEP-3) contained PCR inhibitors. To solved this problem, we check various dilution (1x-100x) of DNA sample, we found that 50x diluted DNA give PCR amplification in short fragment of Cyt b gene (200 bp) but remaining fragments of Cyt b gene were not amplified (Figure 2C).

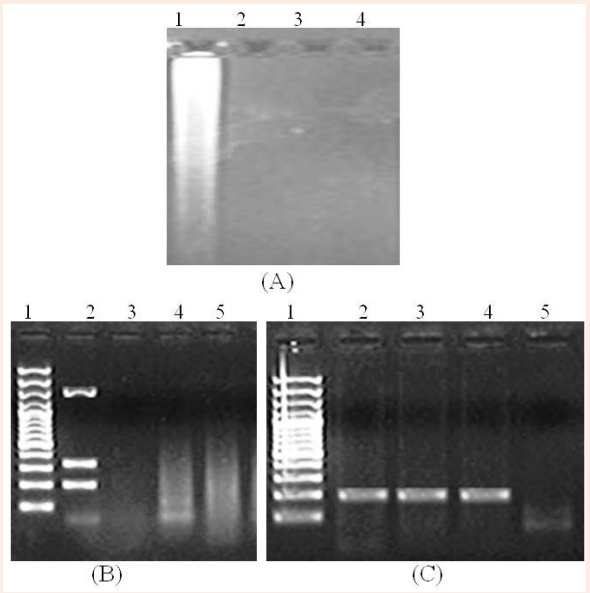

Figure 2: Agarose gel electrophoresis of unknown animal skin sample. (A). Quality of Genomic DNA, Lane 1,2,3 and 4 represent IEP-1, OSP-2, OEP-3 and –Ve control respectively. (B) Amplified PCR product, Lane 2, 3, 4 and 5 indicate IEP-1, OSP-2, OEP-3 and –Ve control respectively. Only Unknown skin sample IEP-1 show amplification in all three different sized primer (200bp, 350bp and 1140bp). (C) Amplification after 50x dilution Lane 2 to 3 are of OSP-2 and OEP-3 respectively. Indicate amplification in 200bp only and remaining primer did not amplified. Lane 4 and 5 are showed +Ve and -Ve control. Lane 1 in (B) and (C) show 100 bp DNA ladder.

Tanning is an aggressive chemical process that potentially damage DNA and this was the probable cause for low yield of DNA in extraction process from the outer parts of the skin sample, which were visibly fully tanned. UV radiation and overexposing to heat are also known to cause DNA damages [12,13] and may have contributed to the negative amplification. The inner ear fragment was probably less damaged by tanning chemicals, UV radiation and heat and resulted in yielding good quality PCR product.

Table 1: List of GenBank accession numbers used in this study and their respective names with geographic origin.

Species/Subspecies	Common Name	Origin	Accessions Number
Cervus albirostris	Thorold's deer	China, Qinghai	AY044863.1
Cervus nippon	Sika deer	China	AB021093.1
Cervus elaphus	Red deer	Germany, Encloser	AY118198
Cervus elaphus hippelaphus	Middle European red deer	Yugoslavia	AY070225.1
Cervus elaphus brauneri	Krim red deer	Ukraine	AY148966.1
Cervus elaphus hippelaphus	Middle European red deer	France	AY244491.1
Cervus elaphus scoticus	Scottish red deer	Scotland	AB021099.1
Cervus elaphus atlanticus	Red deer	Norway, Hitra	AY070226.1
Cervus elaphus xanthopygus	Isubra	Russia, Anjui	AY070224.1
Cervus elaphus canadensis	American wapiti	North America	AF423198.1
Cervus elaphus sibericus	Siberian wapiti	China, Mongolia	AY044862.1
Cervus elaphus kansuensis	Kansu red deer	China, Dong Da Shan	AY070223.1
Cervus elaphus macneilli	MacNeill's deer	China, Qinghai	AY035875.1
Cervus elaphus wallichi	Shou	China, Tibet	AY044861.1
Cervus elaphus barbarus	Barbary red deer	Tunisia, Tunis	AY070222.1
Cervus elaphus corsicanus	Sardinian deer	Sardinia	AY244489.1
Cervus elaphus maral	Maral	Iran	AF489280.1
Cervus elaphus songaricus	Tien Shan wapiti	China, Tien Shan-	AY035871.1
Cervus elaphus hispanicus	Spanish red deer	Spain, La Gaganta	AF489281.1
Cervus elaphus hippelaphus	Middle European deer	Bulgaria	AF423195.1
Cervus elaphus bactrianus	Bactrian red deer	Tadzikistan	AY142327.1
Cervus elaphus yarkandensis	Yarkand red deer	China	AY142326.1
Cervus unicolor	Sambar	India	JN861032.1
Axis axis	Chital	India	JN596156.1
Cervus duvaucelii	Swamp deer	India	EF079830.1
Axis porcinus	Hog deer	Germany	AY035874.1
Cervus eldi thamin	Thamin deer	Thailand	EF079829.1
Rangifer tarandus tarandus	Reindeer	Norway	DQ673123.1

BLAST analysis of Cyt b gene indicated that the samples of unknown tanned animal skin (IEP-1) showed 100% similarity *Rangifer tarandus* .The neighbor-joining phylogenetic tree with other deer subspecies also showed that the unknown skin (IEP-1) is belonged to the *Rangifer tarandus* species, commonly known as reindeer, with a strong bootstrap value of 100% (Figure 3). However, five subspecies of wild reindeer are currently recognized: Barren-ground caribou (*R. t. groenlandicus*), woodland caribou (*R. t. caribou*), Grant's caribou (*R. t. granti*), Peary caribou (*R. t. pearyi*), Dawson's caribou (*R. t. dawsoni*, extinct), and are still widely distributed across northern Eurasia and North America (caribou) [13,14]. Today, almost 50% of the approximate 3,00,0000 reindeer in the Old World are wild animals, and some of this population are semi-domestic (*R. t. tarandus*) which are managed in close coexistence in many areas [15,16].

We compared complete sequence of cytochrome b (1140 bp) gene of unknown tanned skin sample (IEP-1) with three subspecies of wild reindeer and semi-domestic reindeer sequence taken from Cronin et al. [16,17] available in GenBank (Table 2). The Multiple Sequence Alignments displayed 100% similarity with wild reindeer Barren-ground caribou (Table 3).

In order to assess the geographic origin of the unknown sample (IEP-1), we retrieved the n=76 sequences of cytochrome b gene (from NCBI Gene Bank) and found 70 haplotypes in these sequences that represent 56 haplotypes from 3 subspecies of wild reindeer; Grant's caribou, Canadian barren-ground caribou and woodland caribou and 14 haplotypes from different geographic origins of semi-domestic reindeer that were found in 13 herds from 3 regions: Alaska, Russia, and Scandinavia (Table 2).

Table 2: List of Haplotypes and GenBank accession numbers used in this study and their respective names with geographic origin. These all Cyt b sequences taken from Cronin et al. [16,17].

Haplotype	Species Name	Wild/ Semi domestic	Geographical Origin	Accessions number
Hap-01	*R. tarandus*	Semi domestic	Norway	DQ673123.1
Hap-02,03, 04,05	*R. tarandus*	Semi domestic	Alaska/Russia,	DQ673122.1,DQ673127.1, DQ673130.1,DQ673131.1
Hap-06,07	*R. tarandus*	Semi domestic	Svalbard Is, Sweden	DQ673124.1,DQ673125.1
Hap-08,09, 10,11	*R. tarandus*	Semi domestic	Russia	DQ673126.1,DQ673132.1, DQ673133.1,DQ673135.1
Hap-12,13,14	*R. tarandus*	Semi domestic	Alaska	DQ673128.1,DQ673129.1, DQ673134.1
Hap-15,16,17,18, 19,20	*R.t.caribou*	Wild	Canada	AY726672.1,AY726673.1, AY726674.1,AY726677.1, AY726675.1,AY726676.1
Hap-21,22,23,24,25,26	*R.t.groenlandicus*	Wild	Canada	AY726679.1,AY726691.1, AY726705.1,AY726706.1, AY726720.1,AY726729.1
Hap-27,28,29,30,31, 32, 33,34,35 ,36,37,38,39,40,41,42,43,44,45,4 6,47,48,49,50,51,52,53,54,55,56 ,57,58,59,60,61,62,63,64,65,66,6 7,68,69,70	*R.t.granti*	Wild	USA	AY726680.1,AY726682.1, AY726685.1,AY726690.1, AY726697.1,AY726701.1, AY726724.1,AY726730.1, AY726703.1,AY726711.1, AY726712.1,AY726714.1, AY726715.1,AY726718.1, AY726721.1,AY726723.1, AY726728.1,AY726683.1, AY726686.1, AY72689.1, AY726700.1,AY726702.1, AY726684.1,AY726704.1, AY726707.1,AY726719.1 AY726720.1,AY726726.1, AY726687.1,AY726688.1, AY726693.1,AY726696.1, AY726699.1,AY726692.1, AY726695.1,AY726713.1, AY726722.1,AY726694.1, AY726698.1,AY726709.1, AY726717.1

The Alaskan herds represent the geographic range from Siberia, Russia and extended to the Seward Peninsula, Alaska. While the reindeer from the Russian herds represent geographical range restricted to Magadan, district in Siberia and the Scandinavian herds reindeer are represent the Norway population. The sequence of unknown sample (IEP-1) matched with the Hap-21, 22 wild reindeer that represents the Canadian population with 57% bootstrap value and displayed 100% similarity. The remaining reindeer haplotypes have sequences similarity and bootstrap values which were lower, when compared with unknown sample (IEP-1) (Table 3 & Figure 4). The unknown query sequence showed 100% similarity with the haplotype H-21, 22 that has been originated from Canada. Therefore, based on the data of Cronin et al. [16,17] we concluded that the animal skin seized by Indian Customs is of the wild reindeer (*R. t. groenlandicus*) and has been originated from Canada. Wild population of reindeer is Least Concern species in IUCN, listed on Appendix III of the Bern Convention.

Table 3: Similarities in the Cyt b locus between unknown sample (IEP-1) and sequences of reindeer subspecies available in GenBank.

Unknown Sample	Subspecies with the Highest Similarity (Genbank Accession)	Query Coverage (%)	Similarity (%)	Hap/Geographic Origin
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap01/Norway
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap-02,03.04,05 / All from Alaska/Russia
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap-06 / Svalbard Is
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap-07 / Swden
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap-08,09,10,11 All from Russia
IEP-1	*Rangifer tarandus tarandus*	100	99	Hap-12,13,14 / All from Alaska
IEP-1	*Rangifer tarandus groenlandicus*	100	100	Hap-21,22,23,24,25,26/ Canada
IEP-1	*Rangifer tarandus granti*	100	99	Hap-22, 24,27/ USA
IEP-1	*Rangifer tarandus caribou*	99	98	Hap-15,16,17/ all from Canada

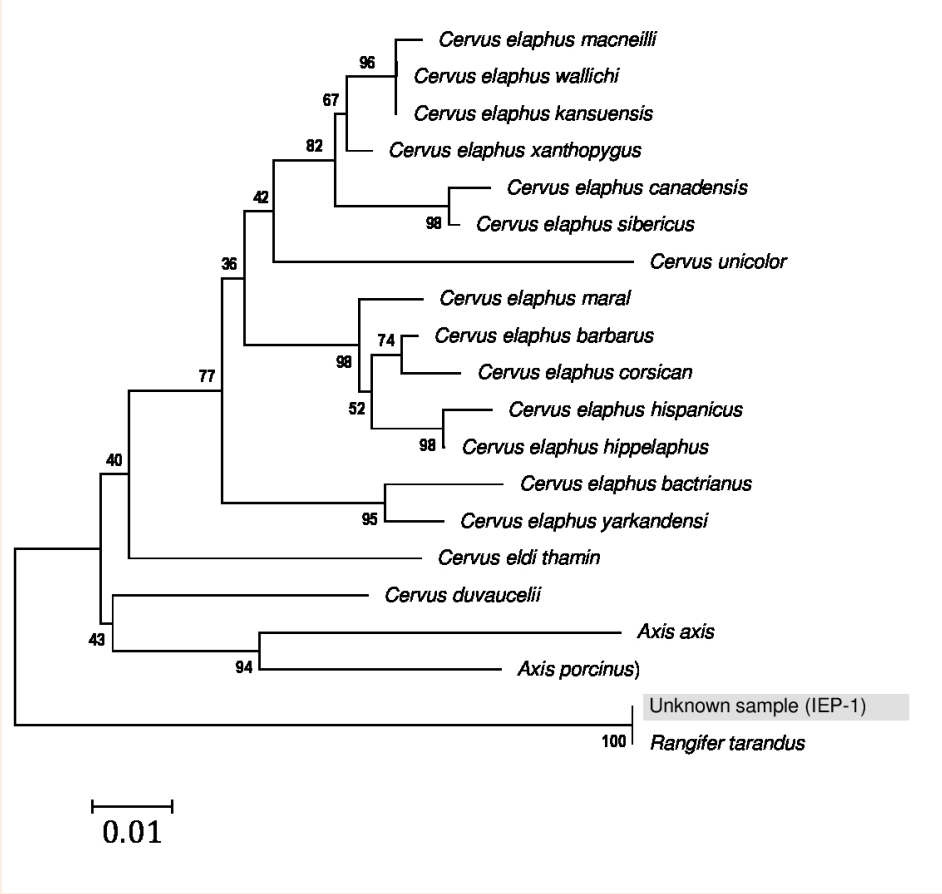

Figure 3: Neighbor-joining phylogenetic tree showing the relationships of unknown skin sample with the other deer species of the world.

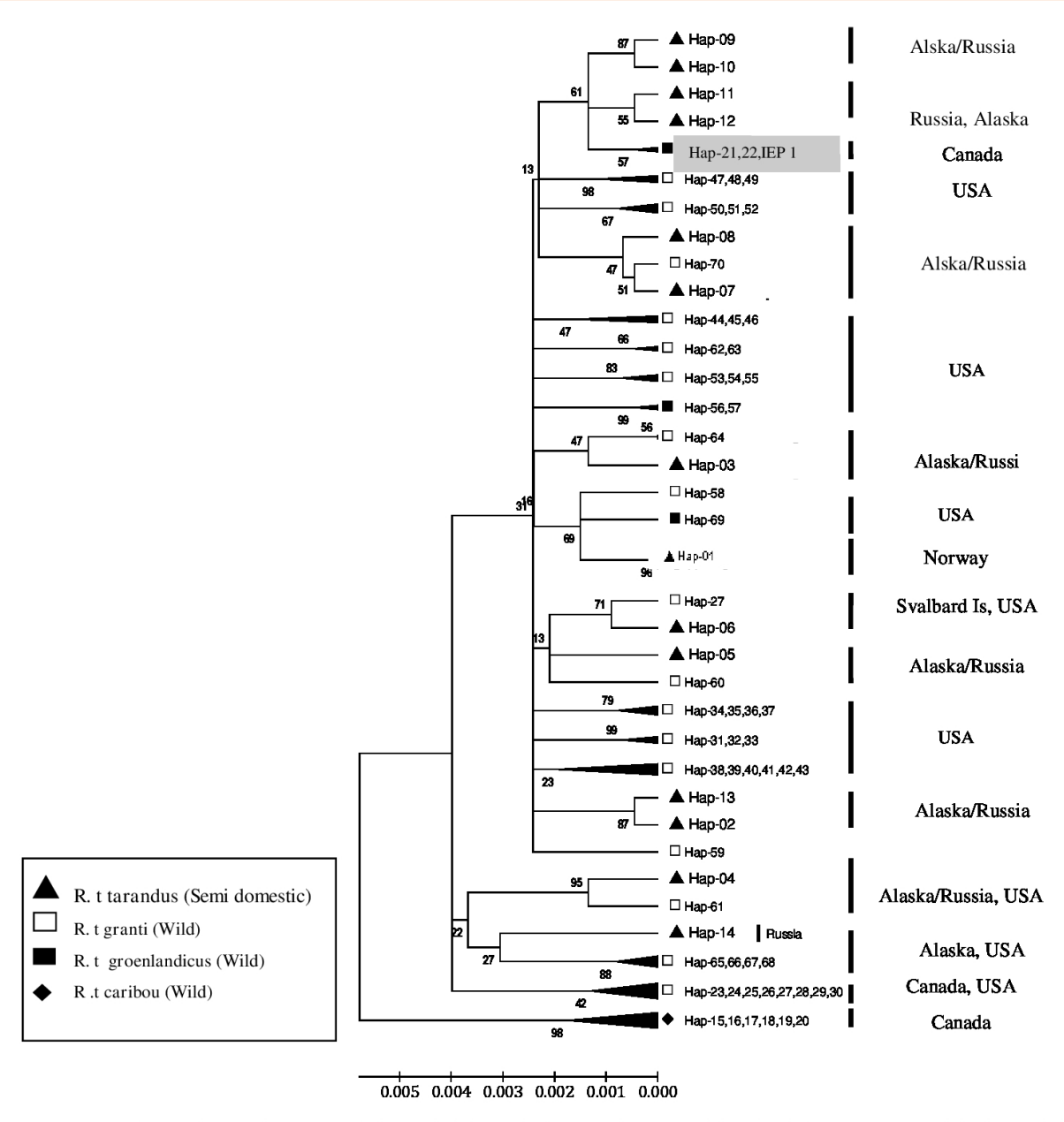

Figure 4: Haplotype Neighbor-joining phylogenetic tree with 70 different geographically originated showing the relationships of unknown skin sample (IEP-1) with the semi domestic (Hap-01 to Hap-14) and three wild subspecies (Hap-15 to Hap-70) of Reindeer. Details of haplotype given in Table 2.

Conclusion

In conclusion, we describe the utility of large fragment of cytochrome b (1140 bp) mtDNA gene sequence in the species identification and ascertaining the geographic origin of a fully tanned animal skin. In order to assign the geographical origin of species, it is necessary to use Cyt b gene, which cover the entire range of probable haplotypes. In many study mtDNA Cyt b gene sequences were used to determine and investigate the geographical origin of species [18,19]. This result shows the importance of genetic analysis in wildlife forensics and the utility of DNA based analysis in the implementation CITES (Convention of International Trade in Endangered Species). We suggest that DNA analysis of tanned skins should target samples from inner

parts of the skins. Furthermore, we defended that database of Cyt b gene sequences across the range of species threatened due to illegal poaching and classified in Appendix I should be established to allow for confident genetic identifications.

Wild reindeer is the least concern species under the IUCN red list. This means that we have a special responsibility to take care of and manage the reindeer in a way that will allow future generations to experience viable population of reindeer. This database would be of great aid in DNA-based investigations of illegal trade, the implementation CITES and the detection of geographic poaching hotspots.

Acknowledgment

The authors are thankful to the Director, Dean and Research Coordinator, Wildlife Institute of India (WII), Dehradun, for their strong support. We would like to thank Nodal Officer of Wildlife Forensic and Conservation Genetic Cell (WFCGC). We would like to thank all the researchers and staff of the Wildlife Forensic and Conservation Genetic Cell (WFCGC), for providing scientific and technical assistance while undertaking the work.

References

1. Ogden R, Dawnay N, McEwing R (1999) Wildlife DNA forensics-bridging the gap between conservation genetics and law enforcement. Endang Species Res 9: 179-195.
2. Branicki W, Kupiec T, Pawlowski R (2003) Validation of cytochrome b sequence analysis as a method of species identification. J Forensic Sci 48(1): 83-87.
3. Mitchell SE, Cockburn AF, Seawright JA (1993) The mitochondrial genome of Anopheles quadrimaculatus species: A complete nucleotide and organization. Genome 36(6): 1058-1073.
4. Guha S, Kashyap VK (2006) Molecular identification of lizard by RAPD and FINS of mitochondrial 16s rRNA gene. Leg Med (Tokyo) 8(1): 5-10.
5. Tobe SS, Kitchener A, Linacre A (2009) Cytochrome b or cytochrome c oxidase subunit I for mammalian species identification an answer to the debate. Forensic Sci Int: Genetics Suppl Series 2(1): 306-307.
6. Linacre A, Tobe SS (2011) An overview of the investigative approach to species testing in wildlife forensic science. Investig Genet 2(1): 2.
7. Berger JW, Rubin PA, Jakobiec FA (1996) Pediatric orbital pseudotumor: case report and review of the literature. Int Ophthalmol Clin 36(1): 161-177.
8. Meyer A (1994) Shortcomings of the Cytochrome *b* gene as a molecular marker. Trends Ecol Evol 9(8): 278-280.
9. Kanita Ouithavon (2009) Molecular Phylogenetic Relationships among Thai Deer (Subfamily Cervinae). Ph.D. Thesis.
10. Hall TA (1999) BioEdit a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98.
11. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28(10): 2731-2739.
12. Sinha RP, Häder DP (1961) UV induced DNA damage and repairman review. Photochem Photobiol Sci 1(4): 225-236.
13. Banfield AWF(1961) A revision of the reindeer and caribou, genus *Rangifer*. Natl Mus Can Bull 17(66): 137.
14. (2011) Designatable Units for Caribou (*Rangifer tarandus*) in Canada. Committee on the Status of Endangered Wildlife in Canada, Ottawa, Canada, p. 88.
15. Syroechkovskii EE (1965) Wild reindeer. Smithsonian Institution Libraries, Washington, DC, USA, pp. 290.
16. Cronin MA, Macneil MD, Patton JC (2006) Mitochondrial DNA and microsatellite DNA variation in domestic reindeer (*Rangifer tarandus tarandus*) and relationships with wild caribou (*Rangifer tarandus granti, Rangifer tarandus groenlandicus*, and *Rangifer tarandus caribou*). J Hered 97(5): 525-530.
17. Cronin MA, MacNeil MD, Patton JC (2005) Variation in mitochondrial DNA and microsatellite DNA in caribou (Rangifer tarandus) in North America. J Mammal 86(3): 495-505.
18. Lee SJ, Jung G, Min MS, Kim CK, Lee H, et al. (2011) Genetic origin identification of Siberian chipmunks (*Tamias sibericus*) in pet shop of South Korea. Animal Cells and System 15(2): 161-168.
19. Koh HS, Wang J, Lee BK, Yang BG, Heo SW, et al. (2009) A phylogroup of the Siberian chipmunk from Korea (*Tamias sibericus barberi*) revealed from the mitochondrial DNA cytochrome b gene. Biochem Genet 47(1-2): 1-7.