FORENSIC SCIENCE JOURNAL SINCE 2002

Forensic Science Journal
2014;13(1):9-14
Available online at:fsjournal.cpu.edu.tw

# DNA typing established as an unambiguous tool for species identification in a dispute case

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Received: January 31, 2014 / Accepted: April 27, 2014

## Abstract

We describe a case study, where DNA typing proved to be an unambiguous tool for species identification in a situation of conflict of opinions between molecular verses morphological characterization. In a suspected wildlife offence case, a hide, flesh and dried blood stain on a wood were confiscated from accuse persons. Blood stain was examined for species identification using DNA sequencing method. It confirms that the blood stain was of a nilgai, *Boselaphus tragocamelus*. Subsequently, the seized hide was morphologically examined by an independent veterinary expert and reported that it would be of a sambar deer, *Rusa unicolor*. Two different opinions from independent agencies created uncertainty to the forwarding authority for filing the legal case in court of law. A portion of same hide and putrefied flesh were re-examined by DNA sequencing which confirmed that the hide and meat were of a nilgai, and not of a sambar.

**Keywords:** Forensic Science, DNA typing; wildlife offence; species identification; nilgai; sambar; putrefied flesh.

## Introduction

Control of wildlife crime is a worldwide challenge for wildlife managers. Accurate wildlife management and enforcement depends upon the identification of victim species. It helps in proper implementation of the wildlife protection laws. Species identification based on DNA sequencing (DNA typing) is one of the reliable protocols available in dealing such cases [1-3]. The conserved genes located on mitochondrial DNA (mtDNA) have potential to be used in sequencing based species identification [2]. Based on conserved regions in mtDNA cytochrome b (cyt b) across species several conserved primers have been developed independently to amplify the target region using single pair of universal primers [1, 3]. This approach has been extensively adopted worldwide for species identification in suspected wildlife offence [4-7]. DNA typing is superlative choice because it can utilize the trace of biological sample from the objects for accurate species identification [5].

Species identification based on hair characteristics is another reliable method and commonly used in the field of wildlife biology for studying food habits and prey-predator relationships [8, 9]. Hides having the fur provide the scope to identify the species based on the hair characteristics [10-13]. This technique has enormous potential in dealing the wildlife offence cases [14]. However, a superficial morphometry could lead to ambiguous result. This study describes an interesting conflict situation, where identification of species was carried out by two independent groups using different protocols. One group examined the case using DNA typing and confirmed poaching of a nilgai (*Boselaphus tragocamelus*). Second group examined the case

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using the morphological characteristics and confirmed poaching of a sambar (*Rusa unicolor*).

Nilgai is a native species of India, Pakistan and Nepal and locally extinct from Bangladesh [15]. Sambar is a native species of south and south-east Asia [16]. Nilgai is least concern and sambar is vulnerable species in IUCN Red List [15, 16] and both are placed in schedule III in Wildlife (Protection) Act, 1973, India [17].

## Material and Methods

### *Case report*

In a suspected wildlife offence case, investigating agency seized 30-35 kg of flesh and one hide of an animal loaded on a wooden cart. The wooden cart was having visible blood stain of victim animal. It was detached from the cart and forwarded to Centre for Cellular and Molecular Biology (CCMB), Hyderabad for identification of victim species. The seized flesh was buried in the soil for natural decomposition. The hide was sent to a veterinary college for species identification using morphological features. After conflict in molecular and morphological report, soil buried putrefied flesh and hide piece was again forwarded to CCMB for DNA typing.

## DNA analysis to establish the identity

### *DNA isolation*

A small scrap of the blood stain was collected from the wooden piece by using a sterile surgical blade. Approximately 100mg putrefied flesh and hide was collected for DNA extraction. Genomic DNA (gDNA) was extracted using phenol and chloroform method [18] with the precautions [5].

### *PCR amplification and DNA sequencing*

The extracted DNA samples were used as a template to amplify a partial fragment of cyt b gene using the universal primers: mcb398 “TACCATGAGGACAAATATCATTCTG” and mcb869 ‘‘CCTCCTAGTTTGTTAGGGATTGATCG’’ [3]. Amplification was carried out in 20 µl reaction volume containing 1 µl of the above DNA solution, 100 µM each of dNTPs, 4 pmol of each primer, 1.5 mM MgCl2, 0.5 units of AmpliTaq Gold (Perkin-Elmer-Cetus, USA), and 1 X PCR buffer (10 mM Tris–HCl, pH 8.3, and 50 mM KCl). The PCR conditions were: an initial denaturation at 95 °C for 10 min, followed by 35 cycles each of denaturation at 95 °C for 45 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. The final extension condition was 72 °C for 10 min. The PCR products obtained were sequenced (ABI Prism 3700, PE-Biosystems) on both strands and the sequence resolved was blasted against databases of NCBI using BLAST program [19].

### *Sequence analysis*

Along with the known nilgai and sambar, an antelope *(Tetracerus quadricornis)* and a bovid, *(Bubalus bubalis)* were selected as closely related taxa for sequence comparison. All the sequences were aligned using program CLUSTAL W [20]. The aligned sequences were tested for best nucleotide substitution model, using MEGA 5 software [21]. The Hasegawa-Kishino-Yano using a discrete Gamma distribution model (HKY+G+I) [22] has the lowest Bayesian Information Criterion (BIC) score among all the models tested. A maximum likelihood (ML) phylogenetic tree was constructed by MEGA 5 [21] at bootstrapping of 1000 replication using the HKY+G+I model (Figure 1).

## Hair microscopy

For reference purpose microscopic hair image of sambar and nilgai was illustrated (Figure 2). Before mounting the whole hair to view medulla it was chopped in to 2-3 cm pieces and treated with xylene for 4 hrs and mounted permanently in Canada balsam [23]. The scales present on the surface of the hair were examined using light microscope by making a cast of scales on a glass slide. The cast of hair was prepared using a thin layer of saturated solution of gelatin in distilled water on the glass slide and hair was then delicately placed on it with one end a bit protruding outside the slide. When gelatine was dried, the hair was plucked out leaving the impressions of the scales on the slide [23]. All the images of hairs were captured in Leica DMR comparison light microscope at 400X magnification (Figure 2).

## Result and discussion

The most significant alignment for the cyt b sequence generated with the DNA obtained from blood

stain (bits score 756, E-value 0.00) was with the cyt b gene sequence of nilgai (GenBank accession no. AJ222679). Based on the primary information, DNA sequences of closely related taxa were compared (Table 1). The sequence comparison identified 107 variable sites (Table 1); however, it differentiated all investigated specimens and species by a minimum of 4 nucleotide variations (Table 2). The cyt b sequence obtained from stains on wooden piece had maximum similarity (99%) with known nilgai. Moreover, ML tree demonstrated a taxonomic closeness of the sequence obtained from blood stains with the nilgai (Figure 1). Final report was communicated to the forwarding agency involved in investigation of this case for further action.

### ***Conflict with morphological report and rectification***

Subsequently, a second opinion report based on the hide morphology was received by the forwarding agency which was examined by a panel of veterinary expert. The morphological report indicated that the hide is of a sambar. A conflicting situation between two different opinions created uncertainty to investigating agency in proper judgment of the victim species and filing a legal case in court of law. Second DNA typing was carried out for same hide and decomposed flesh as describe earlier. The BLAST search again confirmed that the source of both the samples was of a nilgai. Furthermore, cyt b based ML tree confirmed that nilgai was illegally hunted in this case (Figure 1), which was supported by the results of sequence comparison and percentage similarity matrix (Table 1 and 2). Furthermore, comparative hair microscopy images unambiguously differentiate nilgai with the sambar (Figure 2).

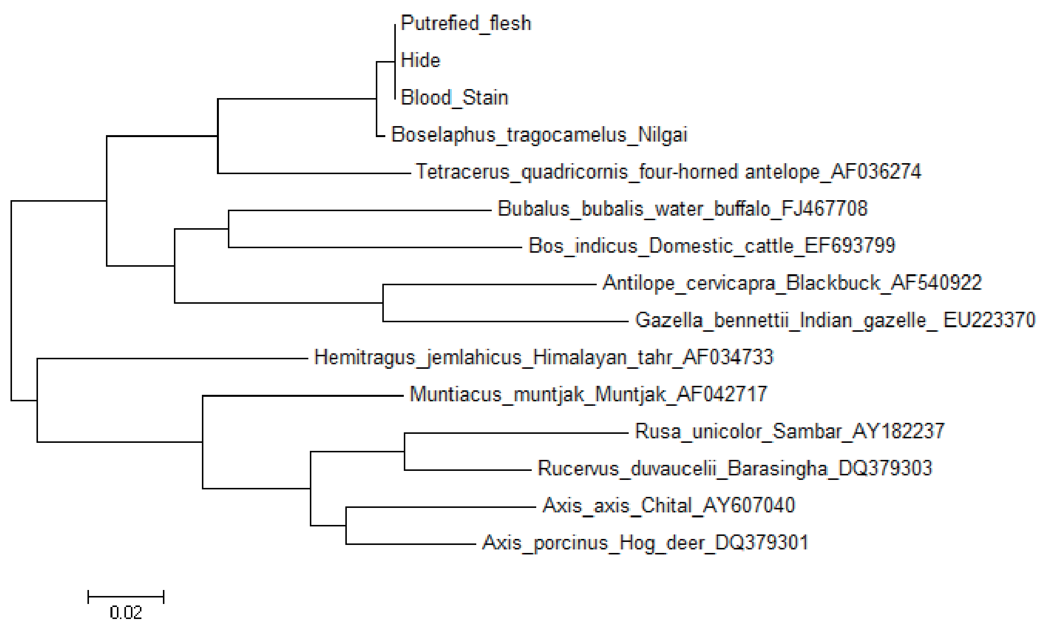


**Fig. 1** Figure 1. ML tree constructed on the basis of partial fragment of cyt b gene demonstrating the relatedness of the case specimens (blood stain, decomposed flesh and hide) with that of nilgai. The GenBank (NCBI) accession numbers of each sequence are given after the common names of the tested species.

(a) (b)

(c) (d)

**Fig. 2** Microscopic hair image (400x) of nilgai (a) cuticle, (b) medulla and sambar (c) cuticle, (d) medulla.

**Table 1**

**Comparision of the cytochrome *b* sequences of blood stain, putrefied flesh and hide with that of diffrent reference animals**

| | |
|---|---|
| | 000000000000000000000011111111111111111111111111111222222222 |
| | 011222234445556667778901112233344555666667778889999112213334 |
| | 436256873694581470692132581703925147136792581470369121262584 |
| Blood stain | ATTTATACCCCACATGACCACCACCTCTCCCCTCCAACTCACCCTAAGTCTTTGCTCATC |
| Putrefied flesh | ............................................................ |
| Hide | ............................................................ |
| *Boselaphus tragocamelus* (AJ222679) | ...............A...G........................................ |
| *Tetracerus quadricornis* (AF036274) | GCCCC..........A......G.TC.C.TT.C.....C.GT....CA.TC..AT..... |
| *Bubalus bubalis* (FJ467708) | GCCCTCC..TTGTG...TG...C..AT....TCAT.G.C..AT.C..AC.CC.A.C..C. |
| *Rusa unicolor* (AY182237) | .C.CTCCTTTT.T.CAG.GCTT.T.C..T.TTC..TGACT...TCG.ACTCCC...TCCT |

| | |
|---|---|
| | 22222222222222222222333333333333333333333444444 |
| | 55566677778888889999000111223566677788899000111 |
| | 03925612350134690139128039581846736925847036289 |
| Blood stain | CTACTCAGCCACTATAGCCAACCATCCCACTGTTCTTGCGTCGCCTC |
| Putrefied flesh | ............................................... |
| Hide | ............................................... |
| *Boselaphus tragocamelus* (AJ222679) | ........................C.......C.............. |
| *Tetracerus quadricornis* (AF036274) | .CG.........C..........TC..T...A....CATA.TATTC. |
| *Bubalus bubalis* (FJ467708) | .C..C.C....TA.C....C.T..C...G.CAC..CC.T.C....CT |
| *Rusa unicolor* (AY182237) | TC.TCTCATTTAAGACTTTTG.T.CGGT.TCACCT.CA.A..AT.C. |

**Number at top represent the variable positions. Identity with first sequence is denoted by dot. GenBank (NCBI) accession number is given in bracket ().**

**Table 2** Comparative table demonstrating number of variable sites (above diagonal) and percent similarity (below diagonal) amongst cyt b sequences generated by universal primers [3] from the specimen no. 1, 2 and 3 with the sequences of reference animals (from NCBI GenBank).

| | Specimens/reference animals | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| 1 | Blood stain | - | 0 | 0 | 4 | 38 | 52 | 80 |
| 2 | Putrefied flesh | 100 | - | 0 | 4 | 38 | 52 | 80 |
| 3 | Hide | 100 | 100 | - | 4 | 38 | 52 | 80 |
| 4 | *Boselaphus tragocamelus* (Nilgai) | 99 | 99 | 99 | - | 38 | 52 | 75 |
| 5 | *Tetracerus quadricornis* (Four-horned antelope) | 90 | 90 | 90 | 90 | - | 55 | 74 |
| 6 | *Bubalus bubalis* (Water buffalo) | 87 | 87 | 87 | 87 | 86 | - | 68 |
| 7 | *Rusa unicolor* (Sambar) | 81 | 81 | 81 | 82 | 83 | 84 | - |

Species identification based on comparison of cyt *b* sequence is an accurate, reliable and reproducible method for confiscated samples [2-5]. This technique was accurately implemented for identification of the species from a minuscule biological sample collected from wooden chopping block [5].

Microscopic examination of hair collected from hide is extremely reliable method for quick identification of the species [8-13]. Both the species has distinct hair morphology at microscopic level (Figure 2), which is in routine use for forensic species identification. Coat colour and size of a mature sambar and nilgai seems to be identical in several situations. If species identification is concluded based on visual morphological features such as coat colour and size without performing microscopic hair characterization it may create a misleading result. In this case, probably veterinary experts concluded species verification report based on coat colour and size without thorough scientific examination, which mislead them. Therefore, while conducting a morphological examination; precautions need to be taken and report should be compiled after proper scientific validation [8-13].

## Conclusions

In this case, cyt b sequences obtained from blood stain, putrefied flesh and hide were 100% (zero variation) similar to each other. These sequences further showed 99% similarity with nilgai, indicating that the sources of specimens were of a nilgai (Table 1 and 2). Whereas, these sequences were only 81% (80 variations) similar with that of a known sambar, which further indicated that these sample could not be of a sambar (Table 1 and 2). It was unambiguously established that all the three biological samples of this case (blood stain, hide and putrefied flesh) were of a nilgai, implying that this animal had been illegally killed. Hence, DNA typing report guided the forwarding authority in accurate filing of the wildlife offence cases in court of law. This study also highlighted that without prior knowledge of a case history DNA typing proven to be accurate, reliable and reproducible tool.

## Acknowledgement

We thank to the Director (Dr. V.B. Mathur), the Dean (Dr. P.K. Mathur) and Nodal Officer Forensic Lab (Dr. Y.V. Jhala) from WII and Dr. S. Shivaji (Retired Group Leader and Scientist) from CCMB for their support. This work was supported by grants from Department of Biotechnology (DBT), Council for Scientific and Industrial Research (CSIR), Wildlife Institute of India, Central Zoo Authority (CZA), Ministry of Environment, and Forest and Climate Change (MoEF&CC), Government of India.

## References

1. Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 1989; 86:6196–6200.
2. Parson W, Pegoraro K, Neiderstatter H, Foger M, Steinlechner M. Species identification by means of cytochrome b gene. Int J Legal Med 2000; 114:23–8.

3. Verma SK, Singh L. Novel universal primers establish identity of enormous number of animal species for forensic application. Mol Ecol Notes 2003; 3:28–31.
4. Hsieh HM, Huang LH, Tsai LC, Kuo YC, Meng HH, Linacre A. JCI Lee Species identification of rhinoceros horns using the Cyt b gene. Forensic Sci Int 2003; 136:1–11.
5. Gupta SK, Verma SK, Singh L. Molecular insight into a wildlife crime: the case of a peafowl slaughter. Forensic Sci Int 2005; 154:214–7.
6. Marin JC, Saucedo CE, Corti P, Gonzalez BA. Application of DNA Forensic Techniques for Identifying Poached Guanacos Lama guanicoe in Chilean Patagonia. J Forensic Sci 2009; 54:1073–6.
7. Tobe SS, Linacre A. DNA typing in wildlife crime: recent developments in species identification. Forensic Sci Med Pathol 2010; 6:195-206.
8. Mathiak HA. A key to the hairs of mammals of southern Michigan. J Wildl Mgmt 1938; 2:251-68.
9. Mayer WVC. The hair of Californian mammals with keys to the dorsal guard hairs of Californian mammals. Amer Midl Nat 1952; 48:480-512.
10. Hausman LA. Structural characteristics of the hair of mammals. Amer Nat 1920; 54:496-526.
11. Brunner H, Coman B. The identification of mammalian hair Shanghai Printing Press Ltd, Hong Kong, 1974.
12. Koppikar BR, Sabnis JH. Identification of hairs of some Indian mammals. J Bomb Nat Hist Soci 1975; 7:35-20.
13. Chakraborty R, Chakraborty S, De JK. Identification of dorsal guard hairs of the species of Indian lesser cats Carnivora: Felidae Mammalia 1999; 65:93-104.
14. Bahuguna A, Mukherjee SK. Use of SEM to recognise Tibetan antelope Chiru hair and blending in wool products. Sci Justice, 2000; 40:177-82.
15. Mallon DP. Boselaphus tragocamelus. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2. <www.iucnredlist.org> 2008; Downloaded on 05 March 2014.
16. Timmins RJ, Steinmetz R, Sagar BH, Kumar SN, Duckworth JW, Islam A, Giman B, Hedges S, Lynam AJ, Fellowes J, Chan BPL, Evans T. Rusa unicolor. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2. <www.iucnredlist.org> 2008; Downloaded on 05 March 2014
17. http://envfor.nic.in/legis/wildlife/wildlife2s3.html Downloaded on 05 March 2014
18. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd edn. New York, NY: Cold Spring Harbor Press, 1989; 40–1.
19. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman J. Gapped BLAST and PSIBLAST: a new generation of protein database search programs, Nucleic Acids Res 1997; 25:3389–402
20. Thompson JD, Higgins DG, Gibson TJ. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22:4673–80.
21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28:2731-9.
22. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 1985; 22:160–74.
23. Bahuguna A, Sahajpal V, Goyal SP, Mukherjee SK, Thakur V. Species identification from guard hair of selected Indian mammals: a reference guide, Wildlife Institute of India, Dehradun, India, 2010 p 85 and 173.