Research Journal of Forensic Sciences ISSN 2321–1792
Vol. **9(1),** 1-6, January **(2021)** *Res. J. Forensic Sci.*

Species level identification of *Lepus nigricollis* from forensic sample using molecular marker and trichology: (First Report)

Soni Neelu[1], Thakare Vijay[2] and Thakare P.V.[1*]
[1]Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, 444602, India
[2]State Forensic Science Laboratory, Amravati, 444602, India
prashantthakare@sgbau.ac.in

Available online at: www.isca.in, www.isca.me
Received 11th March 2020, revised 28th September 2020, accepted 5th November 2020

Abstract

The application of wild life forensics involves identification of species based on the similarities in the DNA sequences of the selective loci. In case of illegal poaching the evidence available are pieces of meat, bones, hairs, blood strains, skin, etc. In the present study pouched samples (meat, hairs with follicle, blood stain and clot) were seized by forest officials but the exact species could not be identified by phenotypic methods. Nucleotide sequence of Cytochrome b (cytb) were 100% similar of all the four seized samples. This revealed that the samples were from same species. The sequences were found to have >90% similarity with other species of Lepus. The exact species could not be identified using Blast analysis but revealed that samples belonged to genus Lepus. Trichological analysis of the seized hairs and comparison with known species revealed that the seized samples belonged to species Lepus nigricollis also called as Indian Hare.

Keywords: Cytochrome b, Trichology, Protected species, Wildlife forensics.

Introduction

Illegal poaching offers finance and attract the attention of international poachers to work in a co-ordinated manner and the annual illegal trade is estimated around US$ 23 million[1]. The adornment and other traditional uses of the body part of wild animals are major challenge to control poaching[2] and threat the survival of critically endangered fauna. Most of the animals are poached for bones, skin, hairs, horns, tusk, etc and the bodies are destroys beyond the scope of identification. It becomes impossible to identify the correct species from the seized forensics evidences[3].

To abide any wildlife crime, it becomes necessary to identify a particular species with confidence. There are mainly two methods, first is DNA based identification and another one is morphological features available in seizers. Use of mitochondrial DNA is one of the most applied methods for identification of species. Cells contain multiple copies of mitochondrial DNA, and can easily be isolated from highly degraded tissue[4]. Examination of the morphological characters of the biological material was one of the rapid modes to deliver a report. The comparison of hair morphometry i.e. trichology of hair is the important way to identify the source of origin of unknown samples[5,6].

Hair is a valuable tool for forensics scientists. It is more resistance to decay than most other body tissues and fluids, thus remain intact for longer time period than other evidence[7]. This durability makes hair one of the most frequently found pieces of evidence at crime scenes[8]. Trichology is useful for identification of species based on samples without recognizable morphological characteristics[9,10]. For microscopic analysis of hair, comparison of the internal structure of the hair is the main tool and is used to identify the exact species from data. The root cells of hair i.e., hair bulb contains DNA that can be used for nucleotide sequence analysis.

It has been noted that most of the mitochondrial locus for many wild animals have not yet studied or their sequences are not available in nucleotide databases, like NCBI, EMBL, BOLD etc. We report a wildlife forensic case study where a combination of trichology and nucleotide sequence helped to solve the case.

Materials and methods

Hair samples, chopper with blood clots, blood strains on cloth and meat was seized by forest officers and were send for analysis to DNA Barcoding laboratory of Department of Biotechnology, Sant Gadge Baba Amravati University (SGBAU), Amravati, Maharashtra (MH) India.

DNA isolation: DNA was isolated using Himedia HiPurA™ Forensic sample genomic DNA purification kit, following the manufacturers' protocol from the following, i. Meat sample, ii. Blood stains on clothand clots on chopper, iii. Hair bulbs of seized sample.

0.8% Agarose gel containing ethidium bromide was prepared for analysis of genomic DNA and were visualized on gel documentation system (Syngene Gbox F3). DNA was stored at 4°C.

PCR Amplification and Sequencing: The PCR amplification was carried out using *cytb* primers[11] that amplified 385bp of DNA. PCR was performed in a 20µl reaction mixture containing genomic DNA, 2µL of 10X Taq buffer, 2µL of 2 mM dNTPs, 1µL of 10pmol/µL each primer, and 2.5 unit of Taq DNA polymerase. The PCR conditions used were initial denaturation of 2:30sec followed by 35 cycles at 94ºC for 30 s, 50ºC for 45s, and 72º C or 45s. PCR products were analysed by electrophoresis on 1.2% agarose gels containing 0.5µg/mL ethidium bromide and were visualized on gel documentation system (Syngene Gbox F3).

The PCR products were purified and sequenced bi-directionally by Sanger`s dideoxy sequencing using ABI 3500 genetic analyser using ABI Big Dye TM Terminator Cycle sequencing kit at Central Instrumentation Cell (CIC), SGBAU, Amravati (MH), India. The forward and reverse sequence were aligned and trimmed at both ends and assembled using Sequencher (Genes codes Corporation, USA).

The sequences so obtained were submitted as query to n BLAST for searching in GenBank for maximum identity and expectation values (E-values). The sequences were aligned using ClustalW and the phylogenetic tree was constructed using Neighbour Joining (NJ) method in MEGA software.

Microscopic studies: Microscopic hair characteristic (light microscopy) for the hair seizers and reference samples were performed according to the protocol provided by Sahajpal[12].

Gross Microscopic Examination: Hair Following parameters of hair were studied with naked eyes as suggested by Taylare and Francis (1999) i. Colour ii. Length iii. Texture iv. Shaft configuration.

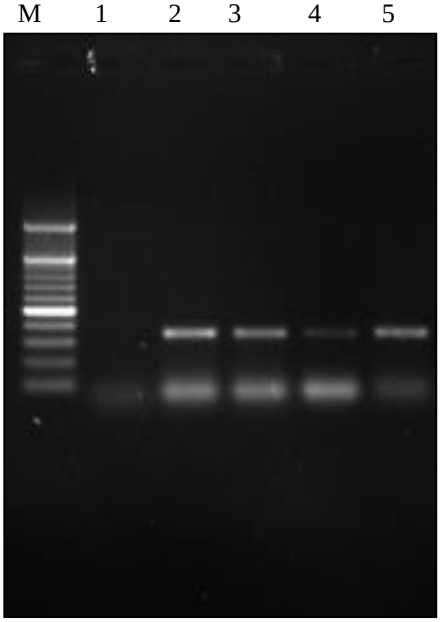
Identifying hair: Identifying whether the hair belongs to which animal is the first step in forensic hair analysis. Animal hairs usually have thick medullae (more than ½ of the hair's diameter). Compare the photos below to what we see under the microscope. Contains photos of *Lepusnigricollis* i.e., Indian hare, these photos are taken from Ph.D. thesis of Milind Shirbhate[13] who studied tricological analysis of various animals under his Ph.D. entitled," Predator-Prey relationship and parasitic infections in wild animals from melghat (Satpuda)." These photos of *Lepus nigricollis* are taken as standard (Figure-3). And these standard reference compared with the photos that we have analysed in the laboratory (Figure-4).

Results and discussion

The samples received by Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati (MH) India were analysed. DNA was isolated from blood clots, blood stains, meat sample and hair bulbs. *cytb* region was amplified from all the DNA samples with an amplified DNA of size ~385bp (Figure-1). The amplified PCR product was sequenced in both directions.

Cyt B nucleotide sequence (359 bases) were aligned using Clustal W from each of the seized sample. *cytb* nucleotide sequences of all the four samples (blood clot, blood stains, meat and hair follicle) were found to be same concluding that they are from same animal species. All the four sequences were compared to the nucleotide sequences described in gene bank using nBLAST alignment tool (Figure-2). The nucleotide sequences under test (contig) did not show 100% match with any of the nucleotide sequences deposited in the genebank. However the sequences showed similarity up to 90% with various species of genus *Lepus.* A neighbour joining tree (NJTree) plotted using MEGA. The NJ Tree showed that nucleotide sequence under analysis (contig) was out grouped and did not matched exactly with any species *cytb* sequences available in database but showed maximum similarly with different species of genus *Lepus* namely *Lepus capensis, Lepus timidus, Lepus sinensis, Lepus mandshuricus and Lepus oiostolus.* The only conclusion that could be drawn at this stage was that the sample belonged to genus Lepus, but the species could not be confirmed.

The hair samples seized from the crime scene were used to study the combination of microscopic hair characters (Figure-4). The hair samples showed particular pattern and was compared with the standard trichology of species studied by shirbhate[13] (Figure-3). From Figure-4 a,b it became clear that the seized hairs matched with *Lepus nigricollis* a protected species under Indian wild life (Protection) act of 1972. It was revealed from *cytb* sequence analysis all four samples were from same source and trichology study revealed that the sample was of *Lepus nigricollis* (Indian hare).



Lane M: 100bp Marker, Lane 1: Negative control, Lane 2: *cytb* from meat, Lane 3: *cytb* from chopper, Lane 4: *cytb* from cloth, Lane 5: *cytb* from hair follicles

Figure-1: PCR amplification of *cytb* gene of ~ 385bp.

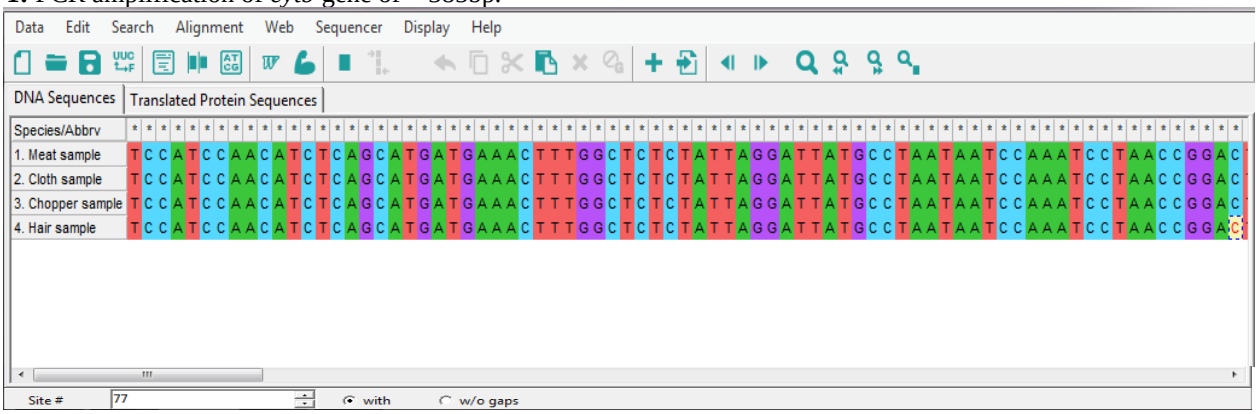


Figure-2: Multiple Sequence Alignment.

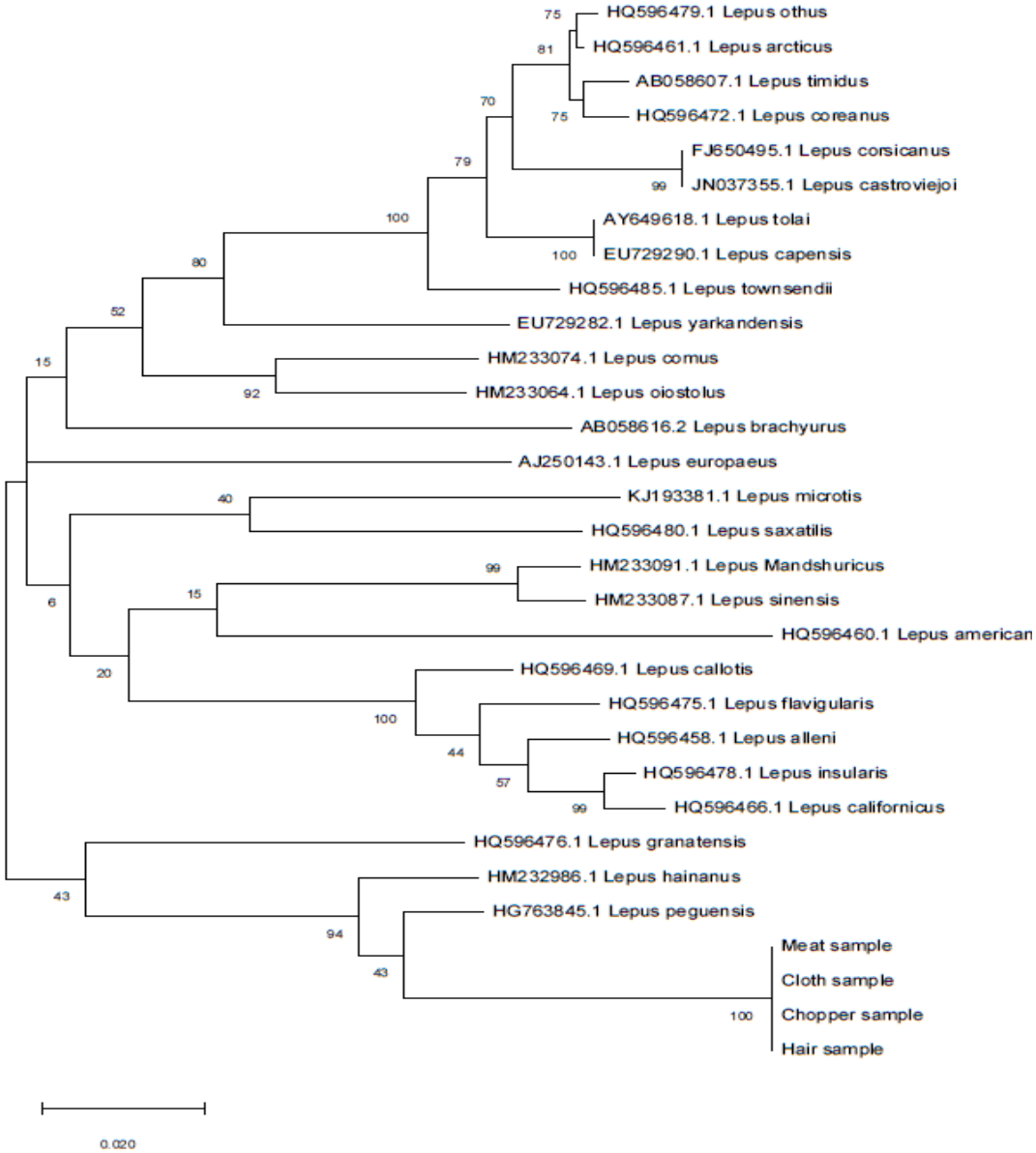


Figure-3: Phylogenetic tree**.**

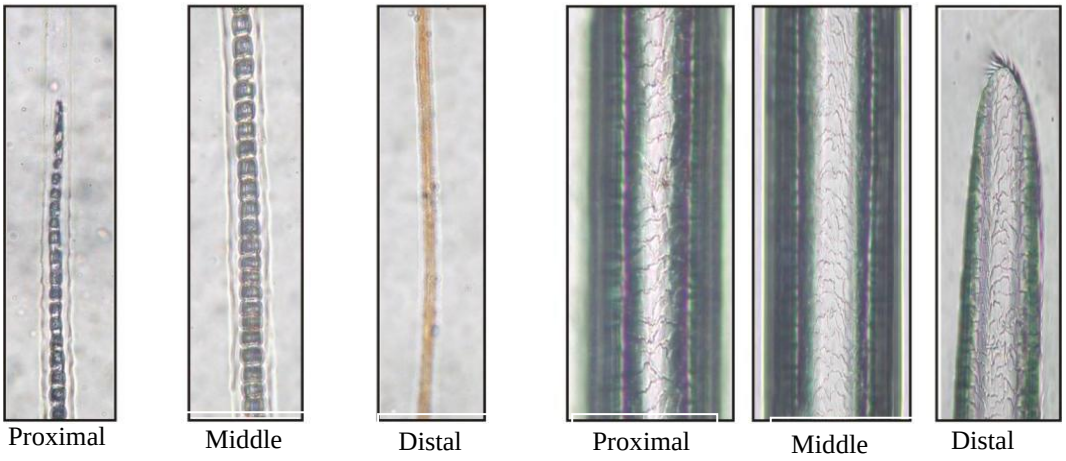
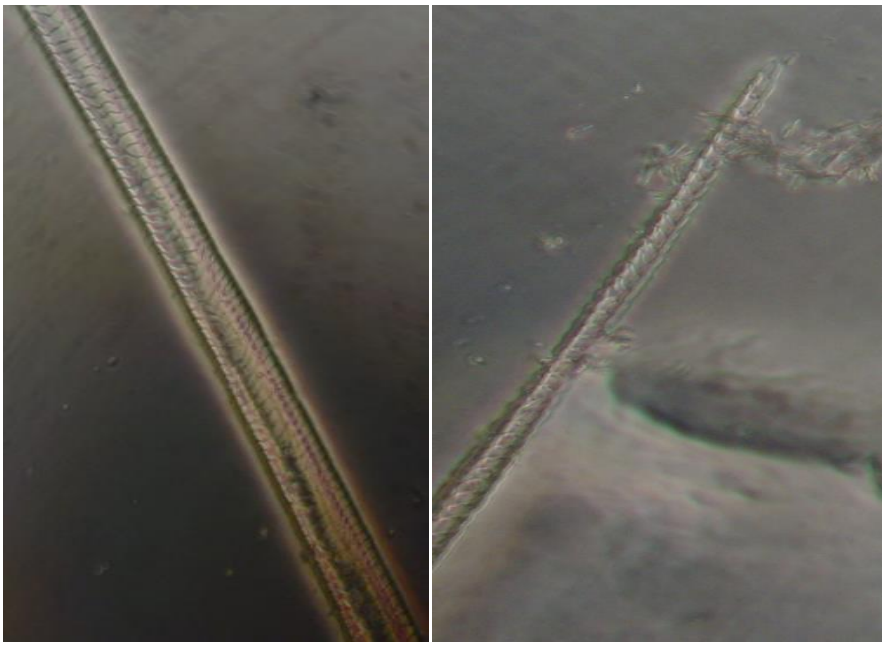


Figure-4: Trichological analysis of *Lepus nigricollis* (Standard)[13].



Cuticular pattern Medullary pattern

Figure-5: Trichological analysis of *Lepus nigricollis (analysed in lab).*

Discussion: For prosecuting a person under the Wildlife protection laws, an exact identification of the poached and seized sample is necessary. It becomes utmost important to identify the species whether it is a protected wildlife species or not. The identification of species using DNA analysis has been an assured technique.

DNA was extracted from hair bulbs, blood clots of chopper, blood stained cloths and meat sample. The contig sequences did not provide 100% species identical matching as there were no voucher specific sequences available. But these sequences showed >90% similarity to the available generic *cytb* sequences and identified genera as *Lepus* for the query under investigation. The *cytb* sequences of other species of genus Lepus are namely *Lepus capensis, Lepus timidus, Lepus sinensis, Lepus mandshuricus and Lepus oiostolus* were present in the database. The sequences from all the four samples were 100% same which lead to conclusion that the samples were from the same animal.

We tried to found out the exact species of *Lepus nigricollis*on the basis of literature and geographical distribution of the species.

Seven subspecies of *Lepus* have been reported from India that included, *Lepus nigricollis aryabertensis Lepus nigricollis nigricollis, Lepus nigricollis simcoxy, Lepus nigricollis ruficaudatus, Lepus nigricollis singhala, Lepus nigricollis*

sadiya, Lepus nigricollis dayanus. The synonyms of *Lepus dayanus* includes *Lepus nigricollis cutchensis, Lepus nigricollis joongshaiensis* and *Lepus nigricollis rajput. Lepus nigricollissimcoxy* includes *Lepus nigricollis mahadeva*[14]. The sub species are distributed throughout India and neighbouring countries.

Lepus nigricollis aryabertensis are endemic to Nepal, while *Lepus nigricollis dayanus* are found in north western region of India from Gujarat, Kutch, Rajasthan, Punjab and extending in Pakistan till Afghanistan. *Lepus nigricollis ruficaudatus* species are the most widely distributed species in India The northern region of India has *Lepus nigricollis ruficaudatus* sub species distributed from Jammu to Arunachal Pradesh till West Bengal except Sunder ban forests and extending towards Orissa, These hares range as far east as Godavari and west as far as Khandesh, Berar and upper Madhya Pradesh region of India and cover the highest geographical area of India. *Lepus nigricollissimcoxy* are restricted to Maharashtra and Madhya Pradesh, while *Lepus nigricollis singhala* are restricted to Sri Lankan island. *Lepus nigricollis sadiya* are restricted to Assam. *Lepus nigricollis nigricollis* are found in the area of southern India extending from Maharashtra to Tamil nadu including Western Ghats[15].

The distribution of various sub species had overlap regions and hence geographical distribution cannot be used as a clear indicator of a particular species. In such scenario hair trichology was performed which is also considered as a common identification tool by wildlife biologist, conservators etc[16].

Hair is known to be a strong evidence for identification as it is resistant to degradation by various environmental factors because of its tough chemical composition[16]. Different species have a characteristic hair microscopic structure that aids in species identification if background data is available for comparison[17]. Most of the cases received by Wildlife Institute of India, Dehradun identifies species using hair[16].

Hair is composed of two parts root and shaft. Root contains the hair follicles that have cells containing DNA which can be utilised for DNA analysis[18]. But in forensics if limited hairs are available with no roots, hair characterisation by microscopy is the more convenient way for identification. Hair shaft consist of outer layer (cuticle), middle layer (cortex) and inner layer (medulla)[19].

The scales are usually classified into three types viz; coronal: completely encircling the hair shaft, spinous: long, narrow and not encircling the hair shaft and imbricate: short, wide and not encircling the hair shaft. The appearance of a medulla is classified as continuous (unbroken), intermittent (regular intervals), or fragmented (irregular intervals)[20]. The *Lepus*hair in lab showed imbricate, flattened scales in cuticle with intermittent multiserial ladder structure in medulla. Similar structure was also observed by Shirbhate[13]. Hence, comparing our images with those standards confirmed that the hair were of *Lepus nigricollis* only.

The study using molecular mitochondrial marker and trichology successfully helped in identifying the seized sample by forensic department.

Conclusion

The hair sample submitted by Regional Forensic Science Laboratory, Amravati was found to be of *Lepus nigricollis* (also called as Indian hare) based on trichology analysis. The tissue sample of meat and the hair samples, blood spotted cloth and chopper were analysed for DNA barcoding. It was found that all the samples belong to same species. Since there was no sequence submitted in NCBI that was matching with the *cyt b* nucleotide sequence, other methods of microscopic analysis of hairs (trichology) was performed, confirming with standard species, it was found that hairs belong to species *Lepus nigricolis.* The samples were from same source (DNA analysis). It was confirmed that the samples were of Indian hare (*Lepus nigricollis*).The sequence of 359bp length has been submitted to NCBI and the accession number provided by them is MT039015.

References

1. Penny Wallace:https://www.traffic.org/about-us/illegal-wildlife-trade/.29/3/2020

2. Johnson, R.N., Wilde, L.W. and Linacre, A. (2014). Current and future directions of DNA in wildlife forensic science. *Forensic Sci Int Genet.*, 10, 1-11.

3. Gupta, S.K., (2012). DNA Wildlife Forensics: Present and Future. *J Forensic Res*., 3(3), 2157-7145.

4. Tobe, S.S. and Linacre A. (2010). DNA typing in wildlife crime: recent developments in species identification. *Forensic Sci Med Pathol.*, 6(3), 195-206.

5. Ogden, R. (2010). Forensic science, genetics and wildlife biology: getting the right mix for a wildlife DNA forensic lab. *Forensic Sci Med Pathol.*, 6(3) 172-179.

6. Linacre, A. and Tobe, S.S. (2011). An overview to the investigative approach to species testing in wildlife forensic science. *Invest Genet.*, 2(2), 1-9.

7. Dalton, D.L., and Kotze A. (2011). DNA barcoding as a tool for species identification in three forensic wildlife cases in South Africa. *Forensic Sci Int.*, 207(1-3), e51-e54.

8. Khan, A., Maryam, J., Yaqub, T., and Nadeem, A. (2014). Human Hair Analysis among Four Different Castes Having Potential Application in Forensic Investigation. *J Forensic Res.*, 5(2), 1-4.

9. Ogden, R., Dawnay, N., and McEwing, R. (2009). Wildlife DNA forensic – bridging the gap between conservation

genetics and law enforcement. *Endang Species Res.*, 9, 179-195.

10. Rastogi, G., Dharne, M.S., Walujkar, S., Kumar, A., Patole, M.S. and Shouche, Y.S. (2007). Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Sci*., 76(4), 666-674.

11. Kocher, T.D., Thomas, W. K., Meyer, A., Edwards, S.V., Paabo, S., Villablance, F.X., and Wilson, A.C. (1989). Dynamics of Mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proc. Nath. Acad. Sci. U S A.*, 86(16), 6196-6200.

12. Sahajpal, V., Goyal, S.P., Singh, K., and Thakur, V. (2009). Dealing wildlife offences in India: Role of the hair as physical evidence. *Int J Trichol.*, 1(1), 18-26.

13. Shirbhate, M.V. (2006). Predator prey relationship and Parasitic infections in wild animals from Melghat (Satpuda). Doctorate Thesis, Department of Zoology, Sant Gadge Baba Amravati University, India.

14. Suchentrunk, F. (2004). Phylogenetic relationships between Indian and Burmese hares (*Lepus nigricollis* and *L. Peguensis*) inferred from epigenetic dental characters. *Mamm Bio*., 69(1), 28-45.

15. Chelmala Srinivasulu and Bhargavi Srinivasulu (2012). South Asian Mammals their diversity, distribution, and status, Springer Verlag (XI). 1-467. ISBN 978-1-4614-3448-1.

16. Oien, C.T. (2009). Forensic hair comparison: Background Information for Interpretation. *Forensic Sci Comm.*, 11(2), 1-16

17. Vivek Sahajpal and SP Goyal. (2018). Species Characterisation from Hair of Protected Mammals: Comparison of Molecular Methods. India, 89-127. ISBN 978-981-13-15831_6

18. Felix, G.A., Piovezan, U., Quadros, J., Juliano, R.S., Alves, F.V., and Fioravanti, M.C.S. (2014). Thricology for identifying mammal species and breeds: its use in research and agriculture. *Arch. Zootec.*, 63(R), 107-116.

19. Vinayak, V., Chitralekha, S.K., Kadyan, A. and Rai, A. (2012). Forensic trichology and its importance in crime cases. *Nat and Sci.*, 10(9), 116-120

20. Deedrick, D. and Koch, S. (2004). Microscopy of Hair Part II: A Practical Guide and Manual for Animal Hairs. *Foren Sci Comm*., 6(3), 1-28.