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FORENSIC EXAMINATION OF GORAL SAMPLES SEIZED UNDER WILDLIFE OFFENCE CASES IN INDIA USING DNA BASED TECHNIQUES

Malay Ashvinkumar Shukla[1,2]*, Bheem Dutt Joshi[2], Ved Prakash Kumar[2], Satish Kumar[1], Anil Kumar Mehta[1], Surendra Prakash Goyal[2]

[1]Laboratory of Forensic Biology and Biotechnology, Institute of Forensic Science, Gujarat Forensic Sciences University, Gandhinagar - 382007, Gujarat, India.

[2]Wildlife Institute of India, Post Box # 18, Chandrabani, Dehradun-248001, Uttarakhand, India.

*(Email Id: malayshukla29@gmail.com)

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Present study is aimed to identify species of Himalayan goral distributed in Indian Himalayan region using mitochondrial Cytochrome Oxidase I gene. Himalayan Goral is in illegal trade for consumption of its meat and skin and horns as trophy material. Few studies depict taxonomic status of Goral and there is no data available based on molecular genetics for Goral species distributed in India. DNA sequences of Cytochrome Oxidase I gene for n=16 samples were generated in the present study and were subjected to NCBI BLAST for similarity and revealed low sequence similarity with Himalayan goral sequences available from the China in the NCBI database. Intra-species sequences divergence for the goral species was in range of 0.001-0.013 and inter-species sequence divergence was in range of 016-0.0134. Neighbour-joining (NJ) based phylogenetic tree, revealed that all the Goral samples generated in present study clustered in the single clade. However, other goral species formed the separate clade. For the first time, DNA Sequences of Cytochrome Oxidase I (COI) were generated for Himalayan Goral species in India and suggests that there are several ambiguity in the sequences of the NCBI database and researchers should be cautious while using reference sequences from NCBI for species assignment and phylogenetics.

Key words: Goral, Phylogenetics, DNA barcoding, Intra-species variation, Wildlife Forensics

Gorals are classified under family bovidae (Sub-family-Caprinae) and species *Naemorhaedus*. The four recognized species of goral (Xiong *et. al*, 2013), are Himalayan goral (*Naemorhaedus* goral), two species of Chinese goral (*N. caudatus* and *N. griseus*), and fourth species being Red goral (*N. bailey*).Globally, illegal and unsustainable trade of wildlife parts and product is a major threat to biodiversity and species are getting extinct at an alarming rate over the past two decades (Challender *et al.* 2015). Gorals are heavily poached for the local consumption of meat, for demand of its fur/hides and horns(Duckworth and MacKinnon, 2008; Yang *et al.* 2013). Besides these, large part of the illegal trade in mountain species goes unnoticed due to lack of proper vigilance. Above all, mountain ungulates like goral also face sever threats from anthropogenic factors like habitat fragmentation and degradation due to increased pastoral activities, disease transmission through livestock grazing, accidental electrocution from fencing, forest fire etc (Vinod and Sathyakumar, 1999). Owing to its declining population trend and threats posed upon, three species of gorals, viz. *N. caudatus, N. griseus* and *N. bailey* are listed as vulnerable under IUCN; whereas *N.goral* is classified as near threatened (Duckworth and MacKinnon, 2008). CITES have listed all four species of goral under Appendix-I so as to restrict illicit trade of these species. Present study is focused on Himalayan goral (*Nemorhaedus goral*) that is distributed in the Himalayan range of India, Pakistan, Nepal and Bhutan.

In order to keep a check on illicit wildlife trade, it is necessary to have a reliable and accurate forensic examination of seized biological material. Standard barcode region, i.e., Cytochrome Oxidase I (COI) has shown a great potential for accurate species identification (Hebert, *et. al,* 2003). Forensic examination of species in trade has been documented recently by many authors, but there have been no records of case investigation of lesser known or elusive mountain ungulates like goral. Present study, addresses the reliability well as efficacy of COI gene in forensic identification of species from seized biological samples of goral in India.

MATERIALS AND METHODS

Reference samples of Himalayan goral were procured from reference repository of Wildlife Institute of India, Dehradun developed through generous donation of samples from various

forest departments in India. During year 2010, few tanned skins were seized in a single consignment by Uttarakhand Forest Department. These skins were screened morphologically and speculated to be of goral. All these confiscated samples were assigned unique identity number based on different consignment. DNA sequences of sub-species of Goral were downloaded from NCBI GeneBank for intra-specific comparison. All the samples were pre-processed and sterilized using absolute ethanol to remove any debris or contamination from environmental DNA. Genomic DNA was isolated from all biological samples (reference and confiscated) using Qiagen D Neasy blood and tissue kit (Qiagen, Valencia, USA) with slight modification as per the need. Quality and concentration of genomic DNA was determined using 0.8% agarose gel electrophoresis and also checked for purity using NanoDrop-1000 spectrophotometer (Thermo Scientific, Lithuania). Due measures were taken to avoid any possible contamination along with incorporation of negative controls as well.

The amplification was performed with COI gene (Folmer et al, 1994) containing reaction mixture of 0.5 U Taq polymerase (Fermantas), 2.5 mM MgCl2, 200 µM dNTPs, 0.5 ?M of each primer, nuclease free waterand 40-50 ng of each DNA in a 20 µl reaction volume on 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The cycling conditions were initial denaturation for 5 minutes at 95°C followed by 40 cycles of denaturation at 95°C for 45 seconds, annealing at 53°C for 1 minute, extension at 72°C for 40 seconds and final extension at 72°C for 10 minutes. The amplified PCR products were visualized under UV-transilluminator for the desired DNA fragment on 2% agarose gel. Purified PCR products were subjected to cycle sequencing reaction using forward using Big Dye® terminator v. 3.1. cycle sequencing kit (Applied Biosystems, Foster City, USA). Obtained products were then further purified using optimized protocol of ethanol-sodium acetate precipitation and pellet obtained were duly suspended in Hi-Di formamide, denatured on thermal cycler and subjected to ABI 3130 genetic analyzer.

Data analysis: The raw DNA sequence of reference samples and unidentified confiscated samples were then separated into pool of different datasets. Concensus DNA sequences were cleaned and edited using BioEdit v 7.0.9.0 software (Hall, 1999). Multiple Sequence Alignment (MSA) and comparison of all the sequences were performed using CLUSTAL W programme as implemented in BioEdit v 7.0.9.0 (Hall, 1999). Sequence divergence between the species based on COI gene was determined using MEGA v. 6.0 software (Tamura *et. al,* 2013). Haplotype diversity (hd) and nucleotide diversity (nd) were calculated using DnaSP v.3.5.1 software (Librado and Rozas, 2009) to assess the level of genetic diversity in Himalayan goral population as compared to other species of goral.

RESULTS AND DISCUSSION

We successfully amplified the COI gene in all the samples of goral and the quality of the sequences was good. After the validation of the sequences, final alignment of nucleotide sequences yielded fragment size of 450 bp COI gene. All these sequences were subjected to NCBI BLAST search that revealed 85-92% pairwise similarity coverage w.r.t reference sequences of Himalayan goral on NCBI database. Reasons for low pairwise assignment may be attributed to mis-representation of reference sequences of goral and closely related species on NCBI, similar to the issue that has been reiterated in previous studies (Negi *et al,* 2016). COI gene fetched high pair-wise genetic distance similarity (10.6-11.3%) between Himalayan goral and Chinese goral (Table 1). However, the sequences available for the Himalayan goral in the NCBI also showed the high genetic distance (11.3-13.4%) compared to DNA sequences generated in this study (Table 1). The reason that can be attributed to the high genetic distance may be the lack of reference DNA sequences available for Goral species inhabiting in India. Further, Himalayan goral species found in India may be a different lineage from the Himalayan goral inhabiting in China. Neighbor-Joining (NJ) phylogenetic trees revealed that DNA sequences of all the goral species from present study were clustered in one clade and supported by high bootstrap value (90%). However, three other species of goral clustered in different clades and showed low bootstrapsupport (<50%) in their respective clades (Fig. 1). Mean intraspecific genetic distance ranged from 0.001 to 0.013 for the sequences of COI gene generated in this study.

Inter-specific distance (p) between all species was within predicted range 0.027-0.154. Himalayan goral supported a monophyletic status (98-100% bootstrap support) based on COI gene. Chinese goral (*N.griseus* and *N.caudatus*) formed a sister clade with each other. Dataset showed that samples of Himalayan goral from northern India formed distinct clade

Table 1: Pair-wise genetic distance generated for closely related ungulate species using Cytochrome Oxidase I gene (450 bp)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	NG																				
2	NG	0.012																			
3	NG	0.122	0.125																		
4	NG	0.122	0.125	0.000																	
5	NG	0.122	0.125	0.000	0.000																
6	NG	0.113	0.116	0.012	0.012	0.012															
7	NG	0.134	0.137	0.027	0.027	0.027	0.035														
8	NC	0.108	0.111	0.037	0.037	0.037	0.030	0.061													
9	NB	0.100	0.102	0.103	0.103	0.103	0.100	0.109	0.094												
10	NB	0.100	0.102	0.103	0.103	0.103	0.100	0.109	0.094	0.000											
11	NGr	0.106	0.108	0.097	0.097	0.097	0.094	0.110	0.095	0.037	0.037										
12	NGr	0.113	0.110	0.012	0.012	0.012	0.005	0.035	0.030	0.100	0.100	0.094									
13	NGr	0.110	0.107	0.015	0.015	0.015	0.007	0.037	0.027	0.102	0.102	0.091	0.002								
14	BT	0.085	0.088	0.111	0.111	0.111	0.102	0.123	0.109	0.086	0.086	0.098	0.102	0.105							
15	BT	0.085	0.088	0.111	0.111	0.111	0.102	0.123	0.109	0.086	0.086	0.098	0.102	0.105	0.005						
16	Hj	0.113	0.110	0.119	0.119	0.119	0.116	0.111	0.123	0.111	0.111	0.129	0.113	0.119	0.106	0.106					
17	OA	0.132	0.135	0.154	0.154	0.154	0.145	0.142	0.139	0.115	0.115	0.146	0.145	0.148	0.092	0.092	0.100				
18	PH	0.129	0.132	0.135	0.135	0.135	0.132	0.136	0.133	0.136	0.136	0.139	0.126	0.129	0.135	0.135	0.114	0.109			
19	PN	0.114	0.111	0.134	0.134	0.134	0.134	0.131	0.141	0.123	0.123	0.148	0.134	0.137	0.088	0.088	0.089	0.109	0.115		
20	PN	0.114	0.111	0.123	0.128	0.128	0.128	0.125	0.129	0.117	0.117	0.154	0.128	0.131	0.094	0.094	0.089	0.097	0.115	0.010	

***NG :*Nemorhaedus goral* (Himalayan goral); NC: *Naemorhedus caudatus* (Chinese goral) ;NB: Naemorhedus baileyi (Red goral); NGr: *Naemorhedus griseus* (Chinese goral); BT: *Budorcas taxicolor* (Takin); PH: *Pantholops hodgsonii (Tibetan antelope*); Hj: *Hemitragus jemlahicus* (Himalayan Tahr) ; OA: *Ovis ammon hodgsonii (Tibetan argali)*; PN: *Pseudois nayur* (Himalayan Blue Sheep)**

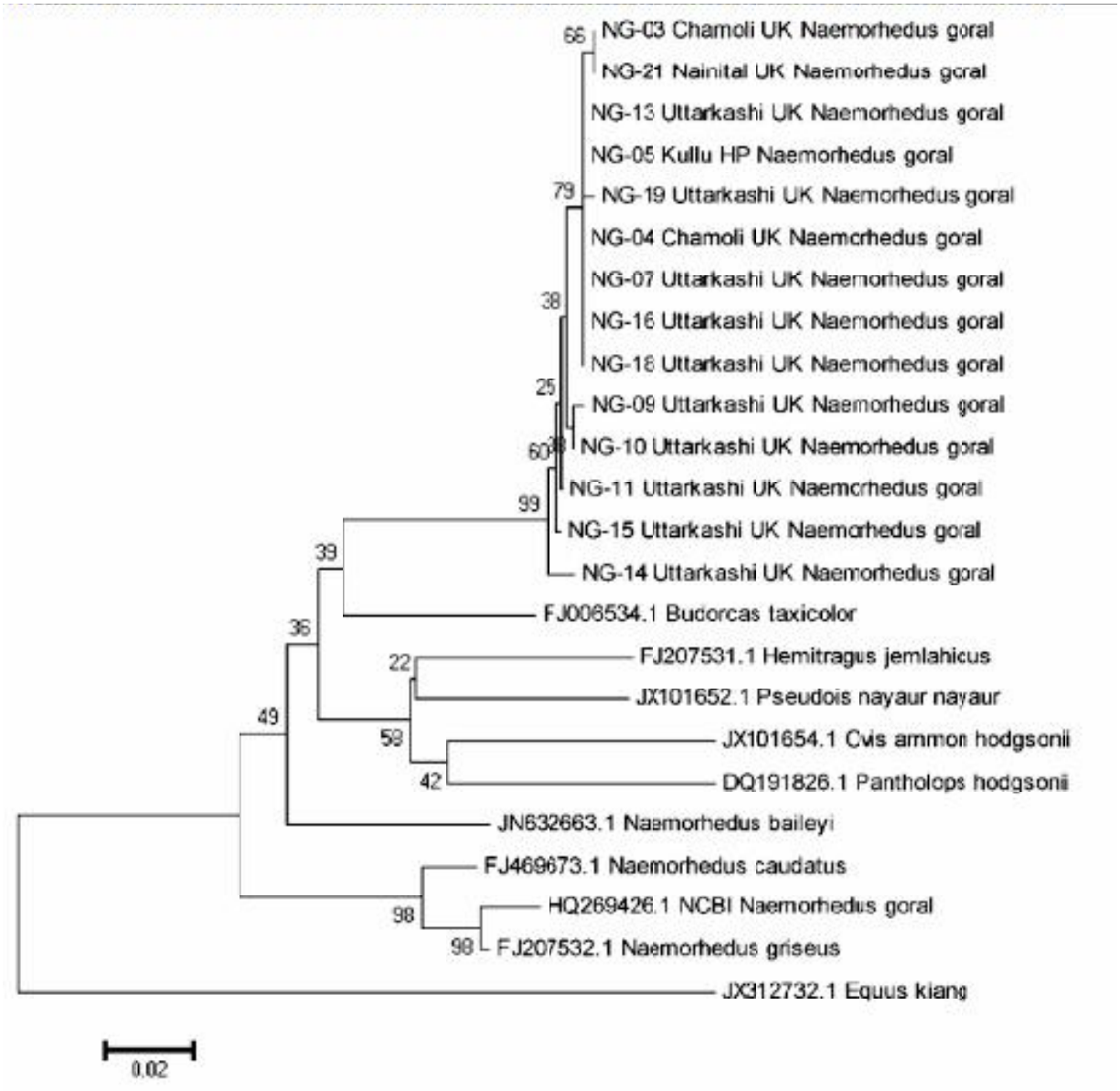

Fig.1: Phylogenetic tree generated using Neighbor-Joining Method for Goral samples used in present study with reference to other wild bovids using Mitochondrial Cytochrome Oxidase-I gene (450 bp).

w.r.t other goral species.

Confiscated skin samples of goral used in present study were genetically assigned to Himalayan goral population based on COI genes with a strong bootstrap support whereas n=5 skins were assigned to barking deer and n=1 skin sample was of otter (Data not shown). This congruence in all the applied approaches enabled us to assign the identity to unknown confiscated samples with accuracy and validity. Values of interspecific divergence estimates of reference samples were higher than 2% which was in congruence with those observed for accurate species distinction in vertebrates using Cytb and COI genes (Hebert *et. al,* 2003; Tobe and Linacre, 2010).

CONCLUSION

Findings from the present study suggests that DNA barcoding can be an effective tool for delineating species from reference samples and from confiscated material during offence cases. Lack of reliable data on Himalayan goral on NCBI GenBank database can lead to poor efficacy of genes in species assignment. Therefore, it is necessary to generate reference genetic database of goral species from India using various mitochondrial genes. Since the wildlife trade is an organized crime, the probable routes connecting the identified hotspots of poaching may be identified and put under vigilance for nabbing the wildlife traffickers. In this way the network of illegal wildlife traffickers can be traced and cracked and the accused may be booked under different wildlife offences.

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Conflict of Interest: Authors declare no conflict of Interest for present study.

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