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Research Paper

DNA barcoding: an identification tool for Indian leopard

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ABSTRACT

Leopard (*Panthera pardus*) is the most widely distributed species of wild cats in the world and extensively involved in man-animal conflicts.This necessitates the identification and monitoring of these wild carnivores in its habitat as well as in buffer zones. Molecular scatology is an approach with great potential for species identification and monitoring of animals in conflict zones at DNA level. In the present study, fecal samples were collected from captive leopards (*Panthera pardus fusca*) (n=8) kept at Arignar Anna Zoological Park, Vandalur, Chennai. DNA was extracted using standard kit protocols. The PCR amplification of *Cytochrome b* gene of mitochondrial DNA (*mt-cytb*) isolated from leopard scat (faeces) generated amplicons of 1067 bp in size. After sequencing and analysis of amplified PCR products, the existence of a highly conserved region 210 bp in length was observed. This conserved region of mt-cytb gene appeared peculiar for Indian leopards (*Panthera pardus fusca*) which depicted considerable nucleotide variations when it was compared with a reference sequence of African leopard (*Panthera pardus pardus*). The sequence can be used as a reference DNA barcode for identification of Indian leopards in forensic studies using DNA extracted from biological materials obtained from the field. The findings of this study suggested that scat samples collected from the field can be used as a reliable, non-invasive and readily available source of genetic materials for species identification of elusive wild carnivores which can aid in demarcation of their territories or movement corridors, to prevent possible human encounters and subsequently reducing the chances of man-animal conflict. The observations of the present study also highlighted the potential of mitochondrial markers like *Cytochrome b* gene to be used for species identification using DNA barcoding. This also happens to be the first time that a DNA barcode has been developed for Indian Leopard (*Panthera pardus fusca*).

Key words: Amplification, barcode, cytochrome b, DNA, leopard, man-animal conflict, scat, sequencing

India is one of the 12 mega-biodiversity nations of the world with an enormously diverse geographical landscape ranging from temperate and arid Himalayas to tropical and sub-tropical plains and is a home to a wide variety of flora and fauna. Wild carnivores belonging to family felidae form an integral part of this biodiversity and are indispensible for maintenance of ecological balance of the nature. There are 36 recognized species of wild cats in the world out of which 15 species are found in India including 5 species of large cats that are tiger (*Panthera tigris*), lion (*Panthera leo*), leopard (*Panthera pardus*), snow leopard (*Uncia uncia*) and clouded leopard (*Neofelisne bulosa*) (Pandey *et al.* 2007).

Leopard (*Panthera pardus*) is the smallest among big cats and is most widely distributed throughout the world. Leopard is reported as the most successful wild carnivore because of special morphological and physiological features that enable it to thrive in almost all kinds of habitats. There are 8 subspecies of leopard in

the world and 3 subspecies have been found in Indian subcontinent which include Nepal leopard (*Panthera pardus pernigra*), Kashmir leopard (*Panthera pardus millardi*) and the Indian leopard (*Panthera pardus fusca*) (Jacobson *et al.* 2016).

The international union for conservation of nature and natural resources (IUCN) has recognized leopard as a threatened species in their red data book and has been quoted in schedule I of the Indian Wildlife Protection Act 1972. Excessive hunting and poaching for fur and other body parts has significantly decreased their numbers in many parts of the world threatening the maintenance of a healthy and genetically viable population (Anonymous 2021). Reliable data regarding abundance and distribution of a particular species in an area are indispensible for success of different managemental measures taken by concerned wildlife authorities to ensure optimum survival of genetically viable population. But most of these strategies and efforts to prevent or mitigate human-carnivore conflict are hampered due to lack of sufficient information occurrence of species in an area, its population dynamics and pattern of their movement or migration from one habitat to another. As a matter of fact failure to identify movement corridors is considered as one of the most important cause of increasing man animal conflict (Jhala *et al.* 2019). A common and recurring problem reported in majority of the cases is inability to identify the problematic carnivore species which is vital for providing insights into behavior and ecology of the animal. Molecular scatology is an efficient non-invasive method of obtaining important information from scat samples of carnivores obtained from field by DNA based studies. The technique of molecular scatology is based on extraction of DNA from fecal samples using standard protocols. Mitochondrial DNA is considered to be the most suitable one for non-invasive genetic studies due to existence of more number of copies per cell than nuclear DNA. The most commonly used regions of mitochondrial DNA include cytochrome b, cytochrome oxidase (I-III), 12S rRNA and 16S rRNA genes which possess some highly conserved regions (Taber *et al.* 1997). Molecular scatology has become increasingly useful in monitoring wild-animals especially elusive carnivore species of which very often fecal material is the only field sample available for analysis. Its relative high efficiency, low cost, increased accuracy and risk reducing attributes have made it an indispensable tool in wildlife management as well as in ecological studies (Chaves *et al.* 2011). Animal scats contain detached intestinal epithelial cells which might provide sufficient amount of genetic material that can be used to identify species of the animal based on a wide variety of criteria. With proper analysis scat samples from animals can be categorized by species, individuals, gender etc. Genetic studies based on scat will provide substantial information regarding a particular species that has been aimed and can be useful in overcoming constraints faced by the management and the conservation agencies. The technique of molecular scatology will fortify prevention and control strategies directed at mitigation of man animal conflicts by assisting in tracking of animals and establishing their movement corridors besides providing vital clues about their occurrence, distribution, density and population structure. Present study was focused on determining the application of molecular scatology technique under Indian conditions. Indian Leopard (*Panthera pardus fusca*) was chosen for the study because of its abundance than other wild cats and its greater involvement in man animal conflicts.

Since no such study has been carried out on Indian Leopard and keeping in view all the above mentioned factors, the study using the scat samples from the leopard was carried out to establish a DNA barcode for Indian leopard (*Panthera pardus fusca)* which can serve as a reference for identification of unknown samples for forensic or scientific studies.

MATERIALS AND METHODS

Study area: The present study was carried out at Arignar Anna Zoological Park (AAZP), Vandalur, a suburb in the southwestern part of Chennai, Tamil Nadu, India. The study was carried out after fulfilling necessary compliances and obtaining due permission from institutional ethics committee. The animal in the present study was Indian leopard (*Panthera pardus fusca*).

Sample collection: The samples were collected from captive leopards reared at Arignar Anna Zoological Park (AAZP) Vandalur, Chennai. Surface scrapings (3-5 grams) of scat samples were taken using sterile surgical blades (BP blades) in labelled sterile plastic containers and immediately transferred to laboratory in ice flask for storage at -20ºC until further processing. A total number of eight samples (n=8) were collected for present study.

DNA extraction: Extraction of DNA was carried out from each sample using QIAamp Fast DNA Stool Mini Kit, which provided fast and easy purification of total DNA from frozen fecal samples. The purified DNA was of high quality and well-suited for use in PCR and other downstream applications.

Extraction protocol: From each sample, 200mg of scat was weighed and transferred into labeled 2ml microcentrifuge tubes and 1 ml inhibitex buffer was added to each tube followed by vortexing, until the sample was thoroughly homogenized. The samples were centrifuged for 1 minute to pellet scat particles. Following this,

new 1.5 ml labeled centrifuge tubes were taken and 25µl of proteinase k was added to each tube. 600µl of supernatant was pipetted out of 2ml tubes and was transferred into new 1.5 ml tubes containing proteinase k. Then 600 µl buffer AL was added to each tube followed by vortexing for 15 sec and incubated at 70ºC for 40 min. After incubation, 600 µl of ethanol (96-100%) was added to lysate, mixed by vortexing and 600 µl of lysate from each tube was transferred into labeled QIAamp mini spin columns followed by centrifugation at 14000 rpm for 1 min. The spin columns were then placed in new 2ml collection tubes and the previous tubes were discarded. Again 600 µl of lysate was charged on each column from respective micro centrifuge tubes followed by centrifugation at 14000 rpm for 1 min. The procedure was repeated until all the lysate from tubes was transferred to spin columns. The QIAamp mini spin columns were then transferred into new collection tubes and 500 µl of buffer AW1 was added to each spin column and were then centrifuged for 1 min at 14000 rpm. The spin columns were again placed in new collection tubes and 500µl of AW2 was added to each tube followed by centrifugation at 14000 rpm for 3 min. Finally the spin columns were placed in new labeled 1.5 ml micro centrifuge tubes and 100 µl of buffer ATE was directly added to membrane of each spin column and were incubated for 10 min at room temperature. The tubes containing spin columns were centrifuged at 14000 rpm for 2 min to elute the DNA and the extracted DNA was stored at -20 degree Celsius until further processing.

Amplification of mitochondrial Cytochrome b (mt-Cytb) gene:

Selection of target gene: For the present study *mt-cytb* gene was chosen, primarily because of the presence of some highly conserved regions within this gene that exhibit maximum inter-species variation and minimum variation between individuals of same species. The mitochondrial DNA also has higher number of copies per cell than nuclear DNA, resulting in higher amplification success rates than nuclear DNA (Taber *et al.* 1997). Based on the earlier reports, partial sequence of the gene was amplified and studied (Chaves *et. al*. 2011).

Obtaining the full nucleotide sequence: National Centre for Biotechnological Information (NCBI) is an online database (*www.ncbi.nlm.nih.gov*) which provides access to biomedical and genomic information. The sequences for Cytochrome b gene were obtained for leopard (*Panthera pardus*) in FASTA format. The accession no. and gene ID of genes are as depicted in Table 1.

Table 1: Accession number and ID of cyt b gene of leopard

GENE	ACCESSION NO.	GENE ID
Leopard *mt- cyt b*	JF720185.1	380306911

Selection of primers: For the amplification of *mt-cytb* gene, a universal forward primer developed by Shanan *et al.* (2008) and species specific reverse primer developed by Salankar *et al.* (2012) were chosen for the present study. The primer sequences employed for PCR amplification are as depicted in Table 2.

Table 2: Sequence of primers and expected PCR product size

PRIMER *mt-cyt b*	SEQUENCE (5'-3')	Product size
Forward Reverse	GACCAATGATATGAAAAACCATCGTTGT GCCACCAATTCACGTCAGGGCT	1067(bp)

Dilution of primers: The primers were synthesised at M/s. Eurofins Genomics, Bangalore and supplied in lyophilized form with the OD values ranging from 6.6 to 24.0. This was diluted as per the instructions in the primer synthesis report to obtain a stock primer solution having a final concentration of 100 pmol/µl. Then the primers were further diluted (1 in 10 dilution) to a working solution of 10 pmol/µl concentration with millipore water (45 µl of millipore water + 5 µl of primer stock solution).

Determination of annealing temperatures: The annealing temperature of each primer set (forward and reverse primers) was obtained by carrying out gradient PCR, based on the melting temperature of the primer set. The temperature which provided a good and specific PCR product yield was chosen to be the annealing temperature of the primer set.

PCR reaction mixture

For Cytochrome b gene: The final reaction volume of 50 µl was made, with the composition as depicted in Table 3.

Table 3: PCR reaction mixture for Cytochrome b gene

S. No.	PCR Reagents	Quantity (µl)
1.	PCR assay buffer (10X)	4.4
2	$MgCl_2$ (1.5 mM – 3.0mM)	5
3	dNTPs (each at 200 µM)	3
4	Primers : Forward (10 pmoles) and Reverse (10 pmoles)	3
5	Taq DNA polymerase (1 unit)	0.6
6	Template DNA	9
7	Nuclease free water	25
	Total	**50**

PCR Protocol: The amplification of Cytochrome b gene in leopards was carried out with the PCR conditions as mentioned in Table 4.

Table 4: PCR conditions for amplication of Cytochrome b gene in Leopard

Step	Process	Temperature	Duration
1	Initial denaturation	95ºC	5 min
2	Denaturation	95ºC	45 sec
3	Annealing	57.5ºC	45 sec
4	Extension	72ºC	1 min
5	Back to steps 2 to 4		35 cycles
6	Final extension	72ºC	7 min
7	Hold	4ºC	Until the samples were removed

Analyzing PCR products by agarose gel electrophoresis: The PCR products were checked on 2 percent agarose gel. The electrophoresis was carried out at a voltage of 70 V and 500 mA current for 50 minutes. The bands developed were observed in a GelDoc (Bio-Rad, USA) system and the images were stored. Upon confirmation of the size of the amplified products, PCR was performed for all eight leopard samples.

Sequencing of PCR Products: The PCR products of *Cytochrome b* gene were sequenced at M/s. Scigenome, Hyderabad. The instrument used for sequencing was ABI 3730XL DNA analyser (Applied Biosystems, U.S.A).

Determination of DNA barcode sequence for leopards: The nucleotide sequences of Cytochrome b gene obtained from leopards along with a reference sequence obtained from NCBI database were analyzed in Bio-edit software, to identify the conserved regions occurring within all the nucleotide sequences. The identified regions should be free from any type of allelic variation among all the nucleotide sequences. The conserved sequences thus obtained were subjected to BLAST analysis to determine their homology with the already existing sequences of different isolates of leopards in NCBI database. The conserved sequence showing highest homology was selected as a DNA barcode sequence for Indian leopard (*Panthera pardus fusca*).

RESULTS AND DISCUSSION

Identification of carnivore faeces at species level has always been a key requirement for all downstream analysis in which scats are the source of information, especially in case of elusive carnivores where faeces is often the only material available for study. Although fecal identification has been historically made based on features like colour, shape, size, scent etc. these approaches are limited and prone to errors. Several molecular methods have been suggested for identification of carnivore faeces e.g. the thin layer chromatography to distinguish fecal bile acids of some distantly related species (Taber *et al.* 1997). But this method is unreliable owing to the intra-species variation of bile acids depending on individual's diet (Quinn *et al.* 1997).

So far the most reliable method of carnivore faeces identification at species level is genetic analysis of genomic DNA extracted from scat samples. The mitochondrial DNA provides more satisfactory and reliable results in species discrimination than nuclear DNA because of (i) higher amplification success rate owing to more number of copies per cell. (ii) tending to have high inter-species variation. (iii) less homoplasy than nuclear DNA. A diverse array of molecular techniques like species-specific PCR amplification, sequencing and PCR-RFLP of various mitochondrial sequences like *mt-cytb* and *16S rRNA* genes are powerful tools for identification of carnivore faeces at species level.

In the present study molecular analysis of fecal samples from leopards was done and the results have been discussed in the light of new findings obtained in the present study and by comparison with the information already published.

PCR Amplification of target sequence: The PCR amplification of target sequence of *mt-cytb* b gene with the selected primers produced amplified products of required size (1067bp). The agarose gel electrophoresis of PCR products revealed the PCR amplicons of desired size without shearing as depicted in Figure 1. Out of 8 samples subjected to PCR, 87.5 percent (7/8) samples were identified as leopard scats based on PCR assay and remaining one sample did not yield any PCR amplification possibly due to degradation occurring during transport, processing or storage leading to changes in primer binding nucleotide sequences. The PCR amplification of target sequence of *mt-cyt b* gene with the selected primers produced amplified products of expected size for leopards i.e 1067 bp in length. The amplification success rate was higher than 60 percent as reported by for carnivores in Brazil (Miotto *et al.* 1997) but lower than 88 percent for faeces of neotropical felids (Roques *et al.* 2011) and even lower than 90.3 percent for carnivore species from temperate forests (Bidlack *et al.* 1997). Therefore exposure to high humidity and warm temperatures as seen in southern India may play a role in degrading the quality of DNA, which inturn would affect the success rate of DNA amplification. In agarose gel electrophoresis of the amplicons of mt-cytb, there wre no non-specific or co-amplifications observed neither any evidence of prey DNA amplification was observed, this result would indicate that *mt-cytb* gene used in this study might have greater specificity and an advantage in terms of species identification, relative to other mitochondrial markers that have been used previously e.g. *cytochrome c oxidase II* gene (Frantzen *et al.* 1998), *12S rRNA* gene (Pandey *et al.* 2007) and *ATP6* gene (Corey, A. 2010) which have encountered non-specific amplifications of prey DNA from scat samples of wild carnivores.

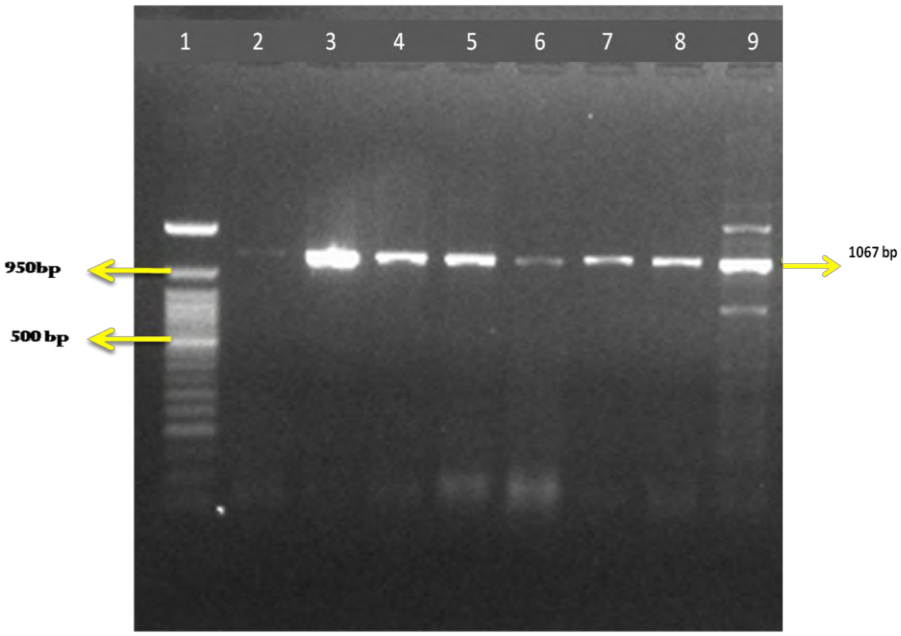


Fig. 1. Agarose gel electrophoresis showing PCR products of *cyt b* gene of leopard. Lane 1: 50 bp DNA ladder; Lanes 2-9: PCR amplicons of *cyt b* gene of leopards

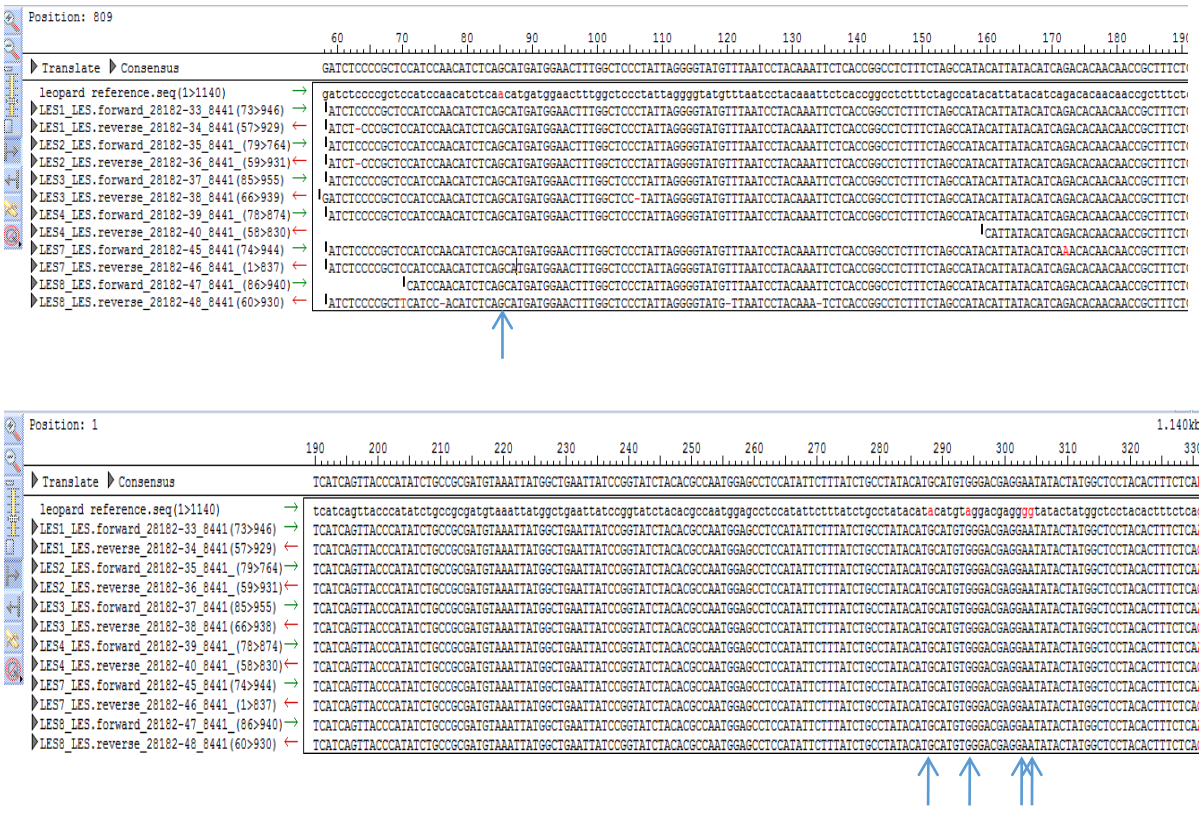
Analysis of Nucleotide sequence of Cytochrome b gene in leopards: The variations found in the nucleotide sequences of Cytochrome b gene among the leopards have been depicted in Figure 2 and Table 5. Out of 8 samples sent for sequencing, 6 samples passed pre-sequencing quality control test. The sequencing success for amplicons of Cytochrome b gene was 75 per cent which is higher than 71 percent for fecal DNA of tropical felines (Michalski *et al.* 2011) but less than 85 percent for fecal DNA from carnivore faeces of temperate regions (Bidlack *et al.* 1997). The sequences were obtained in FASTA and *.abi format and finally a 873 bp

region ranging from nucleotide positions 59 to 932 was analyzed after trimming off noisy ends of the sequences to determine variations and homology in nucleotide positions among these leopards.

Upon analysis, not much variation was found in Cytochrome b gene sequences among Indian leopards. There was very less (< 3 percent) intra-species variation between individuals of same sub-species, this is in line with the findings of Hsieh *et al.* (2001). However variations were found when sequences from Indian leopards (*Panthera pardus fusca)* obtained in this study were compared with a reference sequence of African leopard (*Panthera pardus pardus*) obtained from Genbank. The type of variation encountered was only transition, in which a purine (Adenine or Guanine) was replaced by another purine as seen at positions 85, 288, 294, 303, 304 and 690 and a pyrimidine (cytosine and thymine) was replaced by another pyrimidine, as seen at positions 361, 639, 709, 765 and 774 (Table 5). These variations between Indian leopard and African leopard can be attributed to transversions, substitutions, mutations and deletions of nucleotides during the evolution of these sub-species from their common ancestor. Among the individual leopards 97.3 percent of homology was observed in the partial sequence of Cytochrome b gene. The analysis of partial sequence of Cytochrome b gene adopted in this study was more efficient and less laborious than complete sequencing of gene with little loss of information. The partial sequence will be more successful on highly degrade DNA than sequencing the whole gene.

Table 5: Variations found in the sequence of *cyt b* gene among leopards

Locus (Position in bp)	*Panthera pardus pardus*	*Panthera pardus fusca*						Type of variation
		L1	L2	L3	L4	L7	L8	
85	A	G	G	G	G	G	G	Transition
288	A	G	G	G	G	G	G	
294	A	G	G	G	G	G	G	
303	G	A	A	A	A	A	A	
304	G	A	A	A	A	A	A	
361	C	T	T	T	T	T	T	
639	T	C	C	C	C	C	C	
690	G	A	A	A	A	A	A	
709	T	C	C	C	C	C	C	
765	T	C	C	C	C	C	C	
774	C	T	T	T	T	T	T	



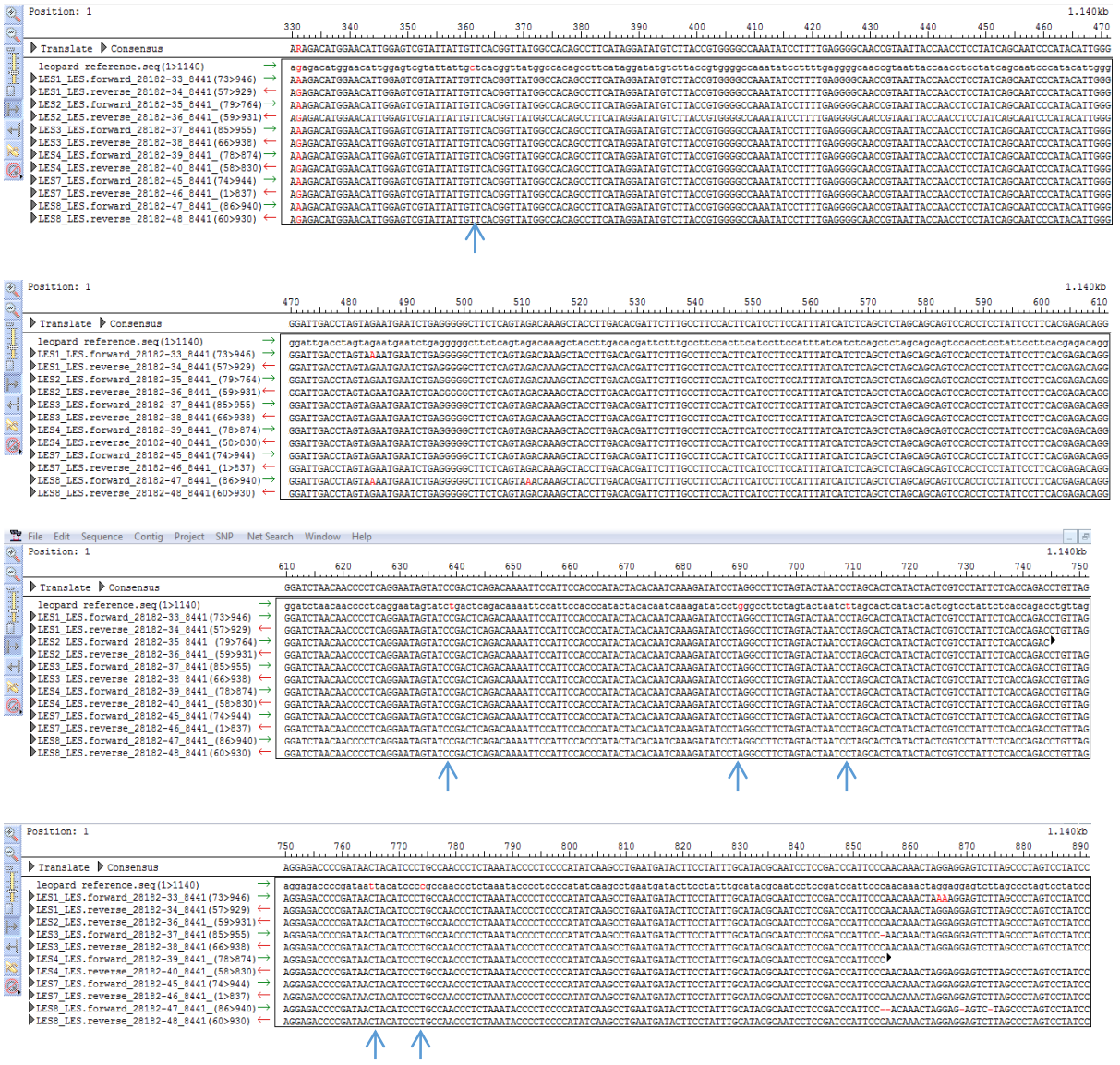


Fig. 2: Analysis of nucleotide sequence of Cytochrome b gene in leopards

DNA Barcode Sequence for Leopard (*Panthera pardus*): During analysis of Cytochrome b gene sequences of leopards, 13 conserved regions were found to exist among all the individuals. The BLAST analysis of these nucleotide sequences revealed a highly conserved region of Cytochrome b gene, 210 bp in length ranging from nucleotide positions 420 to 640 (Plate 3). Hence the nucleotide sequence was selected as the DNA barcode sequence for Indian leopards. The barcode sequence was found to have least intra-species allelic variation among the individual leopards in this study and depicted very high homology ranging from 95-100% with the leopard Cytochrome b isolates available in NCBI Genbank database during BLAST analysis (Plate 4). This short diagnostic segment can be effectively used for identification of unknown samples at species level. This is especially important for identification of biological materials yielding poor quality DNA such as faeces, hairs, museum skins, bones, paleontological remains etc. The findings of this study are in line with the revelations made by Hajibabei *et al.* (2007). The sequence of quite a larger size found as barcode for Indian leopard is also concurred with the qualities of a DNA barcode that are:

(i) Highly conserved among different individuals of same species. (ii) Exhibited maximum homology with isolates from different geographical regions and subspecies of leopards. (iii) The chosen barcode sequence is readily amplified with standard PCR and can be easily detected in sequencing assays (iv) Sufficiently large to be used for identification of species of biological materials containing degraded DNA samples where loss of genetic information is inevitable. The DNA barcode sequence for leopards is as depicted in Table 6.

5'ATTGGAGTCGTATTATTGCTCACGGTTATGGCCACAGCCTTCATAGGATATGTCTTACCGTGG GGCCAAATATCCTTTTGAGGGGCAACCGTAATTACCAACCTCCTATCAGCAATCCCATACATTGG GATTGACCTAGTAGAATGAATCTGAGGGGGCTTCTCAGTAGACAAAGCTACCTTGACACGATTC TTTGCCTTCCACTTCATCCTTCCATTTATCATCTCAGCTCTAGCAGCA-3'

Table 6: DNA barcode sequence for leopards

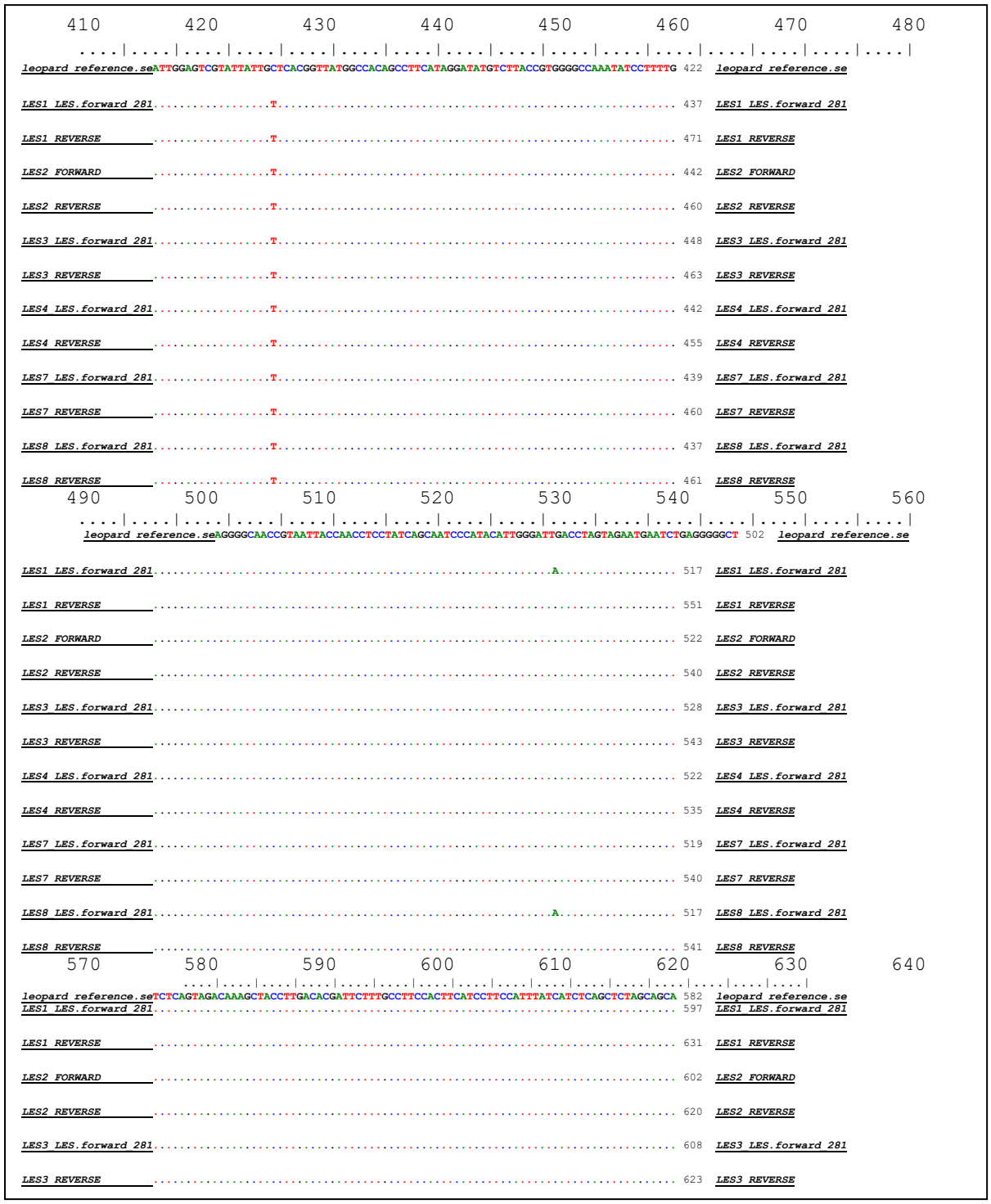


Fig.3: The highly conserved region of Cytochrome b gene among leopards from nucleotide position 420 to 640

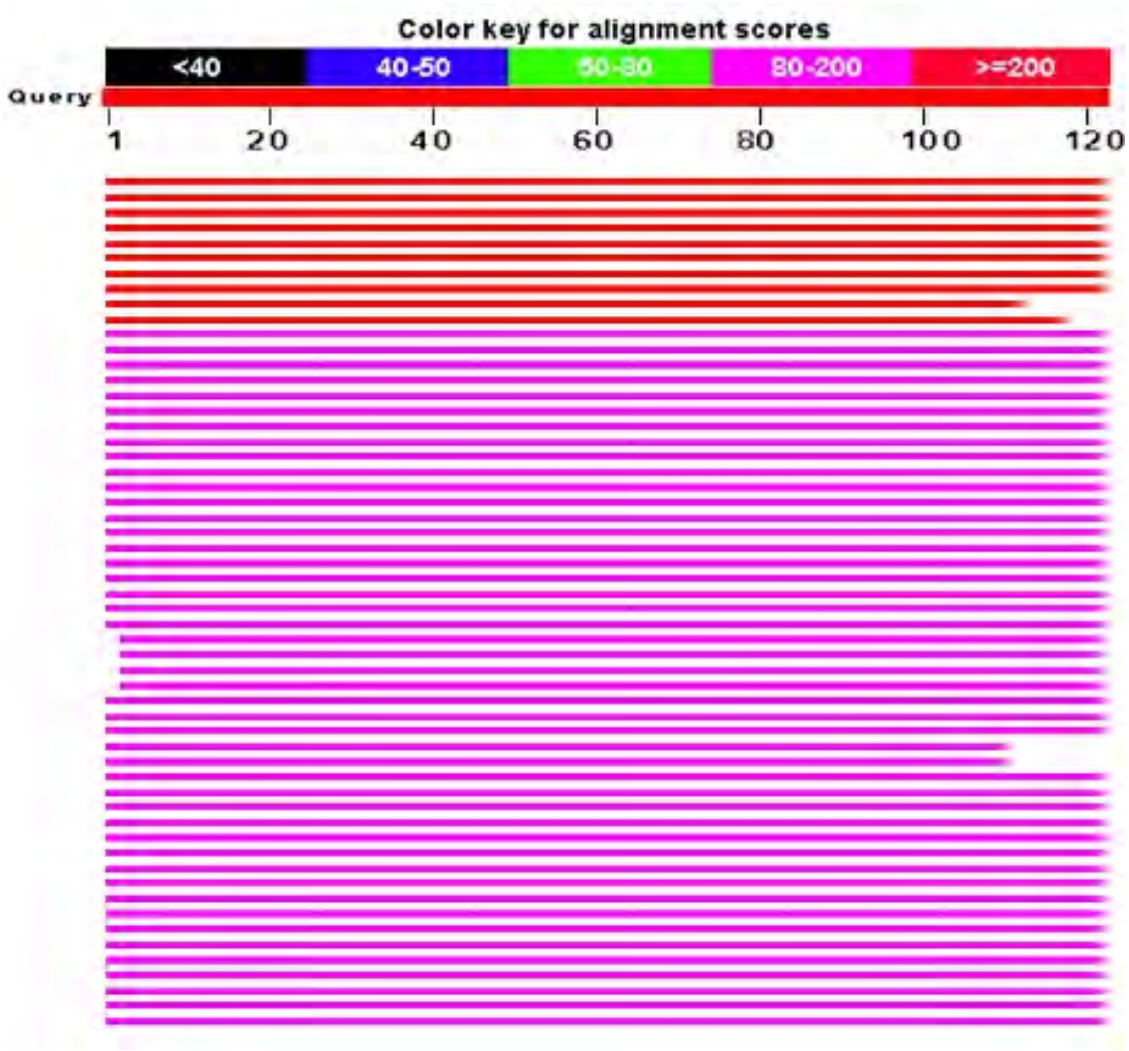


Fig. 4: BLAST analysis of DNA barcode sequence with isolates of Cytochrome b gene of leopards in NCBI Genbank database

CONCLUSION

The molecular scatology is an efficient and reliable non-invasive method for monitoring and identification of species of wild carnivores and can serve as a good alternative to more invasive methods which involve physical capture of animals, risking their health and wellbeing of handlers.

A simple and rapid method for identification of unknown fecal samples collected from the field at species level is PCR amplification of a partial sequence of mitochondrial Cytochrome b gene with species-specific primers, followed by species discrimination on the basis of specific size of the PCR amplicons.

The barcode of 210 bp nucleotide sequence of Cytochrome b gene identified in leopard would serve as a unique sequence for differentiation of biological materials at species level.

The field of molecular scatology demands a holistic approach with introduction of advanced molecular techniques and sophisticated bio informatic tools so that the findings can be further refined to obtain more valuable information.

In future efforts should be made for identification of animals beyond species level e.g. discrimination of an individual animal becomes necessary in certain forensic studies and man-animal conflict related issues, where pin-pointing the exact problematic animal can provide substantial success to preventive strategies adopted.

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