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Research Article

APPLICATION OF PARTIAL CYTOCHROME b and 12S rRNA GENES FOR MAMMALIAN SPECIES IDENTIFICATION

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ABSTRACT

Determination of species from wildlife specimens is one of the prime goals of forensic laboratories analyzing samples involved in wildlife crimes. The conviction rate in wildlife crimes is often low due to a lack of evidential support in identification of the species involved in crime. When species identification by application of morphological techniques fails due to sample autolysis or lack of anatomical markers in wildlife specimens, DNA analysis provides the vital, foolproof cue. Sequences generated from mitochondrial DNA regions are used to determine the species by matching against a known reference sequence, either using the global GenBank database or using references created locally. The present study employed species identification by DNA analysis using wildlife samples received from the Forest Department across Tamil Nadu, India. The study demonstrates the utility of partial *Cytb* and *12S rRNA*genes for determination of species from 18 samples without recognizable morphological features, including tissue, hair and blood. The species of the collected specimens were correctly identified by sequence similarity search with 99 to 100% match and taxonomic classification using phylogenetic tree reconstruction. Of the 18 samples analyzed, accurate species identification using *12S rRNA* gene was possible for all the samples, while *Cytb*gene-based identification was successful for 16 samples. Sequences generated from the study could also serve as a local genetic databank for the State Forest Department to match against sequences from wildlife forensic samples referred to the Institute for ascertaining species involved in wildlife trade. The sequences will also be useful to bridge gaps in genetic data on species native to Tamil Nadu.

Keywords: Wildlife crime, Species identification, mtDNA, Cytb, 12S rRNA.

INTRODUCTION

Determination of species from wildlife specimens is one of the prime goals of forensic laboratories analyzing samples involved in wildlife crimes. The specimens are often meat, hair, ivory, claws, scales, bones or skin from wild mammals, with each sample painting a grim picture of the over-exploitation of wildlife for human monetary gains. Samples from wildlife crime investigations are most often retrieved from illegal hunting, which is one of the major threats to global and national wild vertebrate populations which include several keystone species. The threat posed to wildlife has serious implications on the structure and dynamics of a population in a tropical ecosystem (Kumar *et al*., 2021).

Wild mammals have long been an important resource for humans and have historically been exploited for economic benefits such as food, fibre, fuel and medicine (Boesch *et al*., 2017). Uncontrolled exploitation of wildlife often stems from illegal trade of wildlife and their products, which can drive a species to extinction. To prevent such a scenario, legal frameworks such as the Wildlife (Protection) Act, 1972 and CITES help regulate wildlife trade and enforce wildlife protection laws both nationally and internationally. Despite the existence of strict legislation and penalties, the conviction rate in wildlife crimes is often low due to a lack of evidential support in identification of the species involved in crime. Identification of species, whether protected under law or not, is of pivotal importance in the court of law (Kumar *et al*., 2019).When species

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identification by application of morphological techniques fails due to sample autolysis or lack of anatomical markers in wildlife specimens, DNA analysis provides the vital, foolproof cue. In DNA based species identification, mitochondrial DNA (mtDNA) loci are most often targeted owing to their high copy number, lack of recombination and rates of mutation that coincide with rates of species evolution, thereby allowing efficient discrimination between species (Branicki *et al*., 2003; Jabin *et al*., 2019). The high copy number of mtDNA also accounts for increased sensitivity of analysis, especially when using highly degraded samples or samples with intrinsically low amounts of DNA, such as hair (Branicki *et al*., 2003). Among regions in mtDNA, the Cytochrome *b* (Cytb) and *12SrRNA* regions have been demonstrated to be amplified by PCR under standard conditions (Kocher *et al*., 1989), while *COI*, *16S rRNA* and *ND* are regions that are also commonly used as targets (Nittu *et al*., 2021). Sequences generated from these regions are used to determine the unknown species of the specimen by matching against a known reference sequence, using tools such as BLAST and phylogenetic reconstruction (Dawnay *et al*., 2007). The present study demonstrates the utility of DNA based identification of species using mtDNA markers, from samples without recognizable morphological features, such as tissue, hair and blood. The sequences generated from the study were matched against GenBank database to serve as a local genetic databank to match against sequences from unknown wildlife forensic samples.

MATERIALS AND METHODS

Sample collection

The study was carried out using hair, blood and tissue specimens collected during necropsy of mammals from Tamil Nadu, India. Tissue samples were collected in containers with 70% ethanol; hair samples were collected in air-tight zip-lock polythene bags and blood sample was collected in EDTA coated vacutainer. All samples were stored at -20^0 C until processing for DNA extraction.

DNA extraction

Tissue and hair samples were subjected to digestion in lysis buffer followed by phenol-chloroform treatment. DNA extraction from blood was carried out using sodium perchlorate (Gaur and Reddy, 2017; Bartlett and White, 2003). Negative control was included for every DNA extraction process. Quantification and assessment of quality of extracted DNA was carried out using Nanodrop One spectrophotometer (Thermo Scientific).

PCR amplification

Partial fragments of *Cytb* and *12S rRNA* genes of mtDNA (Kocher et al., 1989) were amplified using Eppendorf Nexus GSX1 Mastercycler. The PCR reaction mixture of 10 µL total volume contained 1X Taq buffer (KAPA Biosystems, SIGMA), 0.25 mM dNTPs, 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.25 U *Taq* DNA Polymerase(KAPA Biosystems, SIGMA) and 1 µL of 10-40 ng/µL DNA template. Thermal cycling conditions included initial denaturation at 95^{0}C for 5 min., followed by 35 cycles of denaturation at 95^{0}C for 30 sec., annealing at 55^{0}C for 30 sec., extension at 72^{0}C for 45 sec. and final extension at 72 ^{0}C for 10 min. PCR positive and negative controls were incorporated in every reaction. PCR products were analyzed by loading 2 µL on 2% agarose gels run using TAE buffer, stained with novel juice stain and visualized under UV transilluminator.

DNA sequencing and analysis

Amplified products were purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The purified products were subjected to bi-directional sequencing with forward and reverse primers using ABI 3730 genetic analyzer (Applied Biosystems, California, USA). Sequences were visualized and edited using BioEdit (Hall, T.A., 1999). Sequence similarity percentage was determined using NCBI-BLAST (Altschul *et al*. 1990). Nucleotide frequencies were determined using Python v3.8.4 (Rossum and Drake, 1995). Phylogenetic tree reconstruction and pair wise distances were computed using MEGA X (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

A total of 18 samples from 15 mammalian species (Table 1) were amplified using *Cytb* (ca. 350 bp) and *12S rRNA* (ca. 450 bp) primers. Sequences generated from specimens by bi-directional Sanger sequencing were assembled using Bio Edit tool, matched against NCBI-BLAST, and submitted to GenBank database to procure accession numbers (Table 2). Specimen sequences matched with that of respective species in NCBI-GenBank with a percentage similarity of 99-100% (Table 2). This demonstrates universal nature and discriminatory power of the primers used for amplifying mtDNA target regions. Phylogenetic tree construction was carried out using Maximum-likelihood method and Hasegawa-Kishino-Yano model with 1000 bootstrap replications (Hasegawa *et al*., 1985, Kumar *et al*., 2018) (Figure 1 & 2). The trees constructed were consistent with the present taxonomic classification of mammals included in this study. Pairwise genetic distance matrix data and mean overall nucleotide base frequencies of the sequences are tabulated in Table 3.

Average read length of the partial *Cytb* fragment was 243 bp. Tree construction for phylogenetic analysis was achieved with 80-100 percent bootstrap support. The samples of *Axis axis* and *Paradoxurus hermaphrodites* used in this study did not yield good quality sequences (nucleotides had q value less than 10). Additional samples of the two species can be evaluated for amplification and good quality sequence generation using the primer pair. Average of interspecific genetic distance of the *Cytb* sequences of 16 mammalian species (Table 4) was calculated to be 0.24. Maximum genetic distance of 0.364 was observed between species *M.silenus* and *P.leo*, while

the minimum genetic distance of 0.071 was observed between species *M.mulatta* and *M.radiata*. All the 18 amplified products yielded good quality sequences. Average read length of the partial *12S rRNA* fragment was 329 bp. Phylogenetic tree constructed classified closely related species under the same node, with an 80-100 percent bootstrap supports. Average of interspecific genetic distance of the 12S sequences of 18 mammalian species (Table 5) was calculated to be 0.156. Maximum genetic distance of 0.263 was observed between species *E.maximus* and *H.indica*, while the minimum genetic distance of 0.014 was observed between species *P.pardus* and *P.leo*.

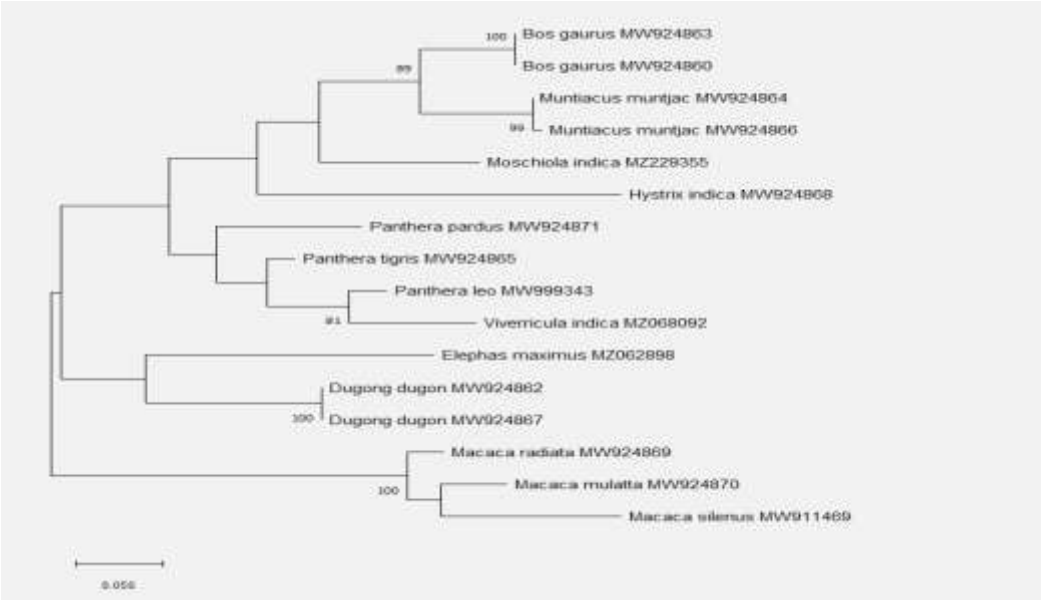

Figure 1. Maximum-likelihood tree of partial *Cytb*gene sequences using Hasegawa-Kishino-Yano model generated from 13 species. Numbers at nodes indicate bootstrap values (values higher than 80 are given).

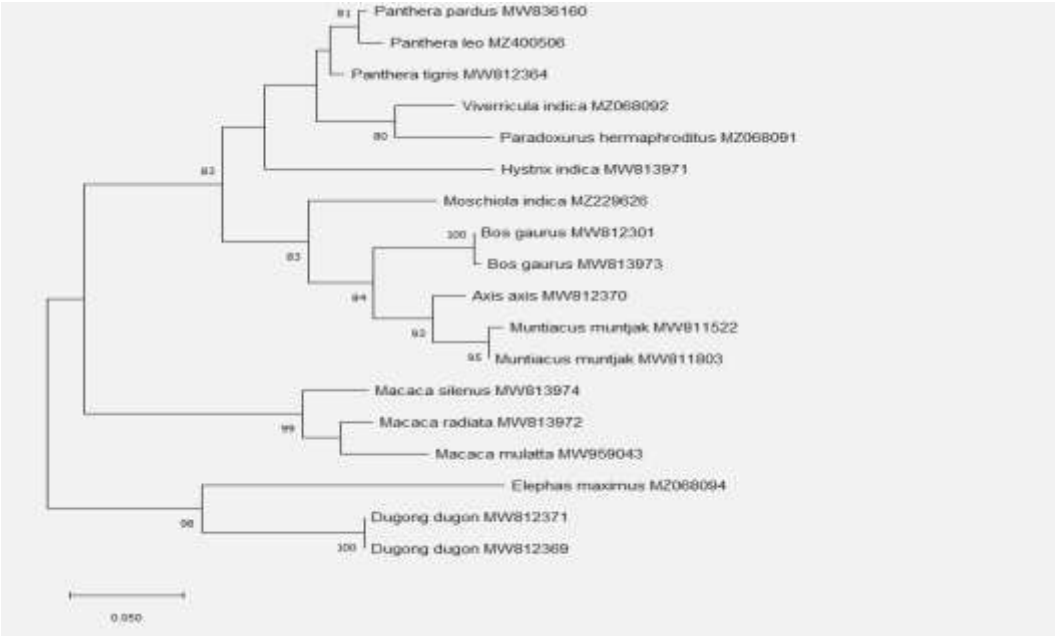

Figure 2. Maximum-likelihood tree of partial *12S* rRNA gene sequences using Hasegawa-Kishino-Yano model generated from 15 species. Numbers at nodes indicate bootstrap values (values higher than 80 are given).

Table 1. Species analysed, its conservation status and significance in illegal wildlife trade (IWT).

Species	Status in WPA	Status in CITES	Significance in IWT	Specimen
Panthera tigris	Schedule I	Appendix I	Poached for tiger skins, bones, claws and teeth (16, 17)	Tissue
Panthera pardus	Schedule I	Appendix I		Tissue
Panthera leo	Schedule I	Appendix I		Blood
Viverricula indica	Schedule II	Appendix III	Traded for production of world's most expensive coffee- Kopi Luwak (18)	Tissue
Paradoxurus hermaphroditus	Schedule II	Appendix III		Tissue
Macaca radiata	Schedule II	Appendix I	Illegal pet trade, hunting & trapping (19, 20)	Hair
Macaca mulatta	Schedule II			Hair
Macaca silenus	Schedule I	Appendix I		Hair
Bos gaurus	Schedule I	Appendix I	Poached for commercial trade in meat and trophies (21)	Tissue
Axis axis	Schedule III	No special status	Poached for skin and meat (22)	Tissue
Muntiacusmuntjak	Schedule III	No special status		Tissue
Moschiola indica	Schedule I	No special status	Poached for meat (23)	Hair
Hystrix indica	Schedule IV	No special status	Poached for meat and quills (Kumar *et al*., 2021)	Tissue
Elephas maximus	Schedule I	Appendix I	Poached for ivory, tail hair (24)	Tissue
Dugong dugon	Schedule I	Appendix I	Poached for meat and oil (25)	Tissue

Table 2. GenBank accession number of specimen sequences and percentage match with GenBank database.

Order	Family	Species	C. Name	Size	12S rRNA Region			Cytb region		
					Size (bp)	% Match with GenBank	GenBank accession no.	Size (bp)	% Match with GenBank	GenBank accession no.
Carnivora	Felidae	*Panthera tigris*	Tiger	1	329	100	MW812364	241	100	MW924865
		Panthera pardus	Leopard	1	342	100	MW836160	283	100	MW924871
		Panthera leo	Asiatic lion	1	327	100	MZ400506	151	99.34	MW999343
	Viverridae	*Viverricula indica*	Small Indian civet	1	333	100	MZ068092	268	100	MZ062899
		Paradoxurus hermaphroditus	Asian palm civet	1	330	100	MZ068091	-	-	-
Cercopithecidae	Primates	*Macaca radiata*	Bonnet macaque	1	326	100	MW813972	211	100	MW924869
		Macaca mulatta	Rhesus macaque	1	323	100	MW959043	203	99.51	MW924870
		Macaca silenus	Lion-tailed macaque	1	330	100	MW813974	278	100	MW911469
Artiodactyla	Bovidae	*Bos gaurus*	Indian bison	2	326	100	MW812301	270	100	MW924863
						99.69	MW813973	286		MW924860
	Cervidae	*Axis axis*	Chital	1	335	100	MW812370	-	-	-
		Muntiacusmuntjak	Barking deer	2	331	100	MW811522	217	100	MW924864
						99.70	MW811803	233		MW924866
	Trangulidae	*Moschiola indica*	Indian mouse deer	1	335	99.70	MZ229626	274	100	MZ229355
Hystricidae	Rodentia	*Hystrix indica*	Indian porcupine	1	324	100	MW813971	232	100	MW924868
Proboscidea	Elephantidae	*Elephas maximus*	Asian elephant	1	324	100	MZ068094	272	100	MZ062898
Sirenia	Dugongidae	*Dugong dugon*	Dugong	2	325	100	MW812371	237	100	MW924862
					324		MW812369	245		MW924867

Table 3. Average nucleotide frequencies and interspecific genetic distances of mammalian species measured using 2 partial mt DNA regions.

Partial mtDNA region	Nucleotide frequency				Interspecific genetic distance	Average interspecific genetic distance
	A %	T %	G%	C %		
Cytochrome b	28.55 ± 2.6	28.41 ± 2.00	14.66 ± 1.48	28.38 ± 3.98	0.071-0.364	0.24
12S rRNA	36.30 ± 2.25	22.87 ± 1.38	17.88 ± 1.33	22.92 ± 2.53	0.014-0.263	0.156

Table 4. Pair-wise distance matrix for *Cytb* sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13
MW924865 *Panthera tigris*													
MW924871 *Panthera pardus*	0.117												
MW999343 *Panthera leo*	0.079	0.152											
MZ068092 *Viverricula indica*	0.141	0.192	0.099										
MW924869 *Macaca radiata*	0.280	0.294	0.325	0.303									

MW924870 *Macaca mulatta*	0.267	0.272	0.325	0.313	0.072							
MW911469 *Macaca silenus*	0.310	0.347	0.364	0.315	0.123	0.128						
MW924863 *Bos gaurus*	0.207	0.221	0.185	0.188	0.265	0.282	0.315					
MW924864 *Muntiacusmuntjak*	0.176	0.197	0.199	0.160	0.271	0.266	0.319	0.101				
MZ229355 *Moschiola indica*	0.178	0.192	0.192	0.244	0.270	0.231	0.315	0.174	0.160			
MW924868 *Hystrix indica*	0.238	0.238	0.232	0.200	0.300	0.308	0.319	0.229	0.197	0.243		
MZ062898 *Elephas maximus*	0.263	0.286	0.272	0.286	0.280	0.308	0.338	0.277	0.250	0.282	0.271	
MW924862 *Dugong dugon*	0.225	0.239	0.232	0.249	0.261	0.262	0.296	0.239	0.229	0.244	0.257	0.216

Table 5. Pair-wise distance matrix for *12S rRNA* sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
MW812364 *Panthera tigris*															
MW836160 *Panthera pardus*	0.024														
MZ400506 *Panthera leo*	0.028	0.014													
MZ068092 *Viverricula indica*	0.078	0.087	0.072												
MZ068091 *Paradoxurus hermaphroditus*	0.089	0.098	0.080	0.069											
MW813972 *Macaca radiata*	0.178	0.171	0.151	0.184	0.178										
MW959043 *Macaca mulatta*	0.180	0.174	0.156	0.182	0.194	0.051									
MW813974 *Macaca silenus*	0.165	0.165	0.143	0.177	0.187	0.055	0.079								
MW812301 *Bos gaurus*	0.124	0.130	0.116	0.129	0.144	0.192	0.176	0.189							
MW812370 *Axis axis*	0.139	0.145	0.123	0.137	0.153	0.179	0.175	0.188	0.080						
MW811522 Muntiacusmuntjak	0.141	0.147	0.133	0.127	0.145	0.186	0.179	0.167	0.083	0.043					
MW813971 *Hystrix indica*	0.128	0.137	0.128	0.139	0.129	0.176	0.182	0.188	0.150	0.143	0.139				
MZ068094 *Elephas maximus*	0.238	0.248	0.238	0.256	0.234	0.227	0.240	0.242	0.235	0.231	0.241	0.263			
MW812371 *Dugong dugon*	0.187	0.206	0.179	0.189	0.180	0.193	0.199	0.201	0.213	0.190	0.201	0.196	0.175		
MZ229626 *Moschiola indica*	0.120	0.123	0.102	0.122	0.128	0.199	0.199	0.180	0.106	0.102	0.098	0.155	0.212	0.213	

Analysis of the partial mitochondrial *Cytb* and*12S rRNA* genes demonstrated multiple conserved and variable sites that helped in inter-species identification, consistent with previous reports (Panicker *et al*., 2019). This illustrates the broad utility of the primers used in analyzing wildlife samples. Overall, the species analyzed in this study are those that are involved in extensive illegal wildlife trade and protected under national and international laws. The genetic data produced could be helpful for determination of species in future wildlife forensic cases and in creating a local genetic database of species and their products that is highly trafficked and traded. Application of a single primer for determination of species in wildlife forensics is often hindered by limitations such as nature of sample, presence of inhibitors in the sample, quality and concentration of isolated DNA. Use of 2 mtDNA markers can help overcome the limitations as samples may amplify when using at least one of the two markers and will also increase the confidence in data obtained for wildlife forensic samples.

CONCLUSION

The study correctly identified the species of the specimens used and demonstrated the potential of molecular markers utilized for application in wildlife forensic sample analysis. This is the first initiative of Advanced Institute for Wildlife Conservation (AIWC) in identification of wildlife samples through DNA based analysis, with which the Institute can support the State Forest Department and other organizations in examination of wildlife forensic case samples.

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