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Case Report

DNA Tools to Establish Species and Individual Identity of Confiscated Biological Samples in Wildlife Related Crime: A Case Report

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Abstract

DNA techniques are prominently used in forensic investigations involving poaching and illegal trade of endangered animals. In the present case study, we could establish the link between crime scene and seized samples successfully using DNA markers. Three persons were caught, while trespassing the forest area near Badmalhera, Madhya Pradesh, India. Range forest officer seized skin and flesh of wild animal from these persons. The officer also found blood stains on stones and leaves at the suspected spot of crime. The samples seized from the accused and those collected from the crime spot were forwarded to our laboratory to identify species and to establish relatedness amongst the samples, if any. Species identification was done using universal primers' for mitochondrial cytochrome b gene. All the forwarded samples were found to be of same species i.e., Indian spotted deer. Further, genotyping with eight polymorphic microsatellites revealed that the samples seized from the accused were similar to blood stains found at the crime spot.

Keywords: Cytochrome b; Individual identification; Microsatellites; Species identification; Wildlife forensics

Introduction

Conservation and protection assumes greater significance in the present scenario because of increased pressure on forest and the monetary value involved in the wildlife trade. India plays a major role in illegal wildlife trade as an importer, exporter and a conduit for wildlife that enters the $25 billion annual global trade [1,2].

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The greatest challenges for enforcement agencies are to detect illegal poaching and trading of wildlife and to obtain concrete evidence for the same. DNA Forensics has become a major tool in wild life crime investigation [3]. DNA analysis is not limited to only species identification but use of DNA for individual identification in wildlife forensic applications has also increased significantly [4]. This involves investigation to determine if two confiscated samples are from the same individual, to determine the pedigree of a particular individual or for questions requiring assignment to population of origin [5,6].

In this case, both mitochondrial cytochrome b (cyt b) and nuclear microsatellites were used to establish the species and individual identity to link the evidences collected from the spot of crime and seized from the accused.

Material and Methods

Case report

Three persons were caught while trespassing through the forest area at Badamalehra range in Madhya Pradesh. Skin and flesh of wild animal were seized from them. The investigating range forest officer also found blood stains on stones and leaves at the suspected spot of crime. Investigation team collected blood scrapings from the spot and forwarded them along with seized flesh and skin to our lab for species and individual identification. The case was registered as CCMB/WLFC-811 and the samples were given sample codes i.e., WL-1751 to WL-1754, where 'WL' stands for Wildlife.

DNA isolation

Blood scraping from the surface of the stones (WL-1751) and leaves (WL-1752) were directly transferred to 500 µl of blood lysis buffer [0.32 mM Sucrose, 10 mM $MgCl_2$, 10 mM Tris HCl, 50 mM EDTA (pH 8.0), 30 µl of 20% SDS, 20 µl of 20 mg/ml Proteinase K]. Piece of Flesh (WL-1753) and Skin (WL-1754) were given PBS (Phosphate Buffer Saline) wash for overnight to remove impurities like soil and dirt particles. After, complete washing, they were finely chopped and transferred to lysis buffer (50 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 30 µl of 20% SDS and 20 µl of 20 mg/ml Proteinase K). The samples in lysis buffer were briefly vortexed and incubated at 52°C for 8 hrs with gentle rotation. After complete lysis, the samples were processed for DNA isolation using the conventional Phenol- chloroform method [7]. The DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantified using NanoDrop 2000™ UV-Vis spectrophotometer. DNA quality was checked by visualizing it in 0.8% agarose gel.

Species identification

The DNA obtained from all the above samples was amplified using the 'Universal Primers' [8] to generate the species-specific molecular signature. The amplification was carried out in 20 µl reaction volume, using 20 ng of template DNA, 100 µm of each d NTPs, 5 pmol of each primer (Bioserve, Hyderabad, India), 1.5 mM $MgCl_2$, 1 X of PCR buffer (10 mM Tris HCl, pH 8.3 and 50 mM KCl). The PCR conditions were: initial denaturation at 95°C for 10 mins, followed by 35 cycles each of denaturation at 95°C for 45 sec, annealing at 52°C for 50 sec

	1	1111111111	1111111111	1111111122	2222222222	2222222222	2222222222	2222222223	3333333333	3333333333	333344
	11122344	4566778890	0011112222	3333444556	6667789900	0112223333	3444444555	5555566666	7788889991	1123334444	5556667778	899900
	4703628736	9878092570	6925891248	0349258170	3698973925	8170391238	9012478012	3467802568	4501690253	6951340369	1251480395	804703
WL-1751	CTCTTTCTAC	TTCCATCTTC	TCTAACTGTG	TCCCCTCAAC	TCATCTTTCC	CTTCTTCTCT	ATTATTCAAT	TAATTCAACA	GCTGATAGTT	CGCCATTCTT	AACGTCATCC	AGACTC
WL-1752	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
WL-1753	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
WL-1754	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Axis *axis**	.....	.....	.....	.....	.....	.....	.....	.....	.....	.A.....	.....	.....
Axis *axis*#	.....	.....T	.....	.....	.....	.....	.....	.....	.....	.A.....	.....	.....
Axis *porcinus**	...CC..CG.	.CTT.C....	.T....C...	A..T.C....	...C...C..	TC....T...	....A.....	CCC..T..T.	A.C...CA..	.A...C....	...A.T..T.	.....
Axis *porcinus*#	...CC..CG.	.C.T.C....	.T....CA..	A..T.C....	...C...C..	TC....T...	....A.....	CCC..T..T.	A.C...CA..	.A.....	...A.T..T.	..T..T
Cervus *unicolor**	TAT..C.C..	......T...	...G..C...	A.T....G..	...C.C..T.	T...C.TCTC	..CTA.A.GC	ACT..T..T.	ATC.GCC..C	.AT..CCTAC	G.TA...C..	.A....
Cervus *unicolor*#	TAT..C.C..	C....CT.C.	..CG..C...	A.T....G..	...C.C..T.	T.....TCTC	G.CTA.A.G.	ACC..T..T.	ATCAGCC..C	.AT..CCT.C	..TA...C..	...TC.
Antilope *cervicapra**	.A.C.CA.CG	C.A.C.TCC.	....CT..C.	...A.....	C.C.TCC.A.	.CCTC.TC.A	GC.CAAT...	.T.AC.CG.T	C.CTGCC...	AA.T.CCA.C	.G.AC.....	C.....
Antilope *cervicapra*#	.A.C.CAC.G	C...T..CC.	....C..AC.	A..GTC....	C.CCTCC.A.	.CCTC.TC.A	GC.CAAT...	.T.AC.CG.T	C.CTGCC...	AA.T..CA.C	.G.AC.....	C..T..
Boselaphus *tragocamelus**	.A.CCCTCCG	CC...C..C.	C...C.C.AA	CT...CT.GT	CT..TC.CAT	.CCT.CT..A	GCCCAA.T..	.C.GC...A.	A.C.CCCCCC	.A.TGC....	.G....GC.T	.....
Boselaphus *tragocamelus*#	.A.CCCTCCG	CC...C..C.	C...C.C.AA	CT...CT.GT	CT..TC.CAT	.CCT.CT..A	GCCCAA.T..	.C.GC...A.	A.C.CCCCCC	.A.TGC....	.G....GC.T	....C.

Table 1: Comparisons of cytochrome b sequences generated from the seized samples with the sequences of reference animals of major Indian ungulate species involved in wildlife related crime in India. Only variable sites are shown. (Number at top represents variable positions).

*Reference sequences from In-house database

#Reference sequences obtained from NCBI Genbank (JN093092.1, AY035874.1, FJ556575.1, AF022058.1, and AJ222679.1)

and extension at 72˚C for 1 min. The final extension was at 72˚C for 10 mins. PCR products were visualized in 2% agarose gel along with 100 bp DNA ladder (Invitrogen™, USA) to confirm the correct amplicon size. All successfully amplified PCR products obtained were sequenced (ABI 3730) on both strands in triplicates and sequences were aligned using the program Sequencing Analysis 5.2 and Codon Code alligner. Statistical confidence of sequence similarity within and among species was further assessed using a Basic Local Alignment Search Tool (BLAST) [9]. The sequence resolved was compared using MEGA 6.0 [10] with the molecular signatures of the vast number of known animal species (available in database of molecular signature generated and maintained by CCMB) and sequences of closely related Indian species available in mitobase of NCBI. All the aligned sequences for the cyt b showed the maximum sequence similarity with the cyt b sequence of *Axis axis* i.e., Indian spotted deer (Table 1).

Individual identification

After species identification, all the DNA samples along with one positive control (DNA of a known Indian spotted deer) and one negative control (No DNA) were subjected to PCR amplification using 8 microsatellite loci. All the 8 loci (Ca13, Ca18, Ca30, Ca38, Ca42, Ca60, Ca71 and Ca75) are deer-specific [11]. PCR amplification was carried out in 20 µl of reaction mixture containing 30 ng of genomic DNA, 1XPCR buffer, 100 µM dNTPs, 1.5 mM $MgCl_2$, 5 pmoles of each primer, and 0.5 units of amplitaq gold (Applied Biosystems, USA). The reaction conditions were: initial denaturation at 95˚C for 10 min, denaturation at 94˚C for 45 s; annealing at 51˚C to 61˚C for 50 s; and extension at 72˚C for 1 min (30 cycles) with final extension at 72˚C for 7 mins. Forward primers were fluorescence labeled. The PCR reaction was repeated three times and visualized in 2% agarose gel along with 100 bp DNA ladder (Invitrogen™, USA) to confirm the correct amplicon size. All amplified PCR products were size fractionated and visualized on ABI 3770 DNA sequencer. The allele size was determined using Genemapper™ software version 3.1 (Applied Biosystems, USA). The multi-locus genotype profiles of all the samples were subjected to relatedness testing by calculating Likelihood Relatedness (LR) using ML-relate software [12]. The multi-locus genotypes of other known individual Indian spotted deer were also used to compare the LR values.

Results and Discussion

In the present study, high molecular weight DNA was obtained from all the four samples namely WL-1751, WL-1752, WL-1753 and WL-1754. The cyt b sequences obtained for all four samples were aligned and compared with cyt b sequences of the vast number of known animal species in CCMB database and closely related Indian species i.e., *Axis axis, Axis porcinus, Cervus unicolor, Antelope cervicapra* and *Boselaphus tragocamelus* available in

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	WL 1751	0	0	0	0	1	2	33	34	50	53	58	60	63	64
2	WL 1752	100	0	0	0	1	2	33	34	50	53	58	60	63	64
3	WL 1753	100	100	0	0	1	2	33	34	50	53	58	60	63	64
4	WL 1754	100	100	100	0	1	2	33	34	50	53	58	60	63	64
5	Axis axis	99.8	99.75	99.75	99.75	0	1	32	33	49	52	57	59	62	63
6	*A. axis* (JN093092.1)	99.5	99.51	99.51	99.51	99.8	0	33	34	50	53	58	60	63	64
7	*Axis porcinus*	91.9	91.85	91.85	91.85	92.1	91.85	0	5	49	49	68	66	58	59
8	*A. porcinus* (AY035874.1)	91.6	91.6	91.6	91.6	91.9	91.6	98.77	0	52	52	72	65	61	62
9	*Cervus unicolor*	88.6	88.64	88.64	88.64	88.9	88.64	87.9	87.2	0	14	67	67	73	74
10	*C. unicolor* (FJ556575.1)	87.7	87.65	87.65	87.65	87.9	87.65	87.9	87.2	96.5	0	65	63	68	67
11	*Antelope cervicapra*	85.9	85.93	85.93	85.93	86.2	85.93	84.2	83.2	84	84	0	14	55	56
12	*A. cervicapra* (AF022058.1)	85.4	85.43	85.43	85.43	85.7	85.43	84.69	84.9	84	84	96.5	0	56	57
13	*Boselaphus tragocamelus*	84.7	84.69	84.69	84.69	84.9	84.69	86.17	85.4	83	84	86.4	86.2	0	1
14	*B. tragocamelus* (AJ222679.1)	84.4	84.44	84.44	84.44	84.7	84.44	85.93	85.2	82.7	84	86.2	85.9	100	0

Table 2: Number of variable sites (above diagonal) and percent similarity (below diagonal) amongst cytochrome b sequences generated by universal primers from the seized samples (1,2,3,4) with reference sequences of closely related Indian species from CCMB in house database (5,7,9,11,13) and NCBI database (6, 8,10,12,14).

mitobase of NCBI (Respective accession numbers were JN093092.1, AY035874.1, FJ556575.1, AF022058.1, and AJ222679.1), using MEGA 6.0. Sequence comparison shows total 116 variations in total (Table 1) and pairwise comparisions differentiated all investigated species by a minimum of 33 nucleotide variations and the sequences obtained from seized samples showed maximum similarity (>99%) with *Axis axis* i.e., Indian spotted deer (Table 2).

Further, eight pairs of microsatellite loci were successfully amplified in all samples and showed the presence of same allele for each locus (Table 3). The relationship calculated between four samples by estimation of LR value was 1.00, which indicates that the blood stains on stones, leaves and samples seized from the accused belonged to same individual animal. For reference, the LR values between the four samples and other Indian spotted deer available in CCMB in-house database was 0.00, which proves the significant similarity at the level of microsatellite loci. Based upon the forensic evidences obtained, the accused were taken into custody under Indian Wildlife Protection Act (1973) for wildlife poaching and cruelty towards wild animals. We could detect the crime more effectively and establish a link between the evidences and the suspect with a significant degree of confidence. This particular case report once again emphasizes the role of DNA tools in the fight against poaching of endangered and protected species.

Locus	WL-1751		WL-1752		WL-1753		WL-1754	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Ca13	210	218	210	218	210	218	210	218
Ca18	128	128	128	128	128	128	128	128
Ca30	301	307	301	307	301	307	301	307
Ca42	128	150	128	150	128	150	128	150
Ca38	172	190	172	190	172	190	172	190
Ca60	320	320	320	320	320	320	320	320
Ca71	311	327	311	327	311	327	311	327
Ca75	190	202	190	202	190	202	190	202

Table 3: Observed allele size in Base Pair (bp) in four confiscated samples.

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