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CASE REPORT

CRIMINALISTICS

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Species Identification from Dried Snake Venom*

ABSTRACT: Illegal trade in snake parts has increased enormously. In spite of strict protection under wildlife act, a large number of snakes are being killed ruthlessly in India for venom and skin. Here, an interesting case involving confiscation of crystallized dried snake venom and subsequent DNA-based species identification is reported. The analysis using the universal primers for cytochrome b region of the mitochondrial DNA revealed that the venom was extracted from an Indian cobra (*Naja naja*). On the basis of this report, the forwarding authority booked a case in the court of law against the accused for illegal hunting of an endangered venomous snake and smuggling of snake venom. This approach thus has immense potential for rapid identification of snake species facing endangerment because of illegal trade. This is also the first report of DNA isolation from dried snake venom for species identification.

KEYWORDS: forensic science, wildlife forensics, snake venom, species identification, mitochondrial DNA, cytochrome b

Illegal trade in snake body parts, especially skin and venom, has increased manifold in recent times (1). After ivory, snake venom is the most sought after smuggled good in the international market (http://www.traffic.org). A large number of snakes are killed for skin and venom in India. The Indian Wildlife Protection Act of 1972 protects all snakes with high priority for the Indian cobra (*Naja naja*). However, the enforcement agencies fail to implement the law because it is difficult to identify the species from dried snake venom without a complete biochemical analysis.

DNA analysis is the only alternative method for species identification from dried snake venom (2,3). However, there is only a single report available for DNA isolation from dried snake venom, wherein the authors used a commercial kit-based approach and reported a significantly low yield of DNA (3). In the present study, a conventional approach for DNA isolation combined with the "universal primers"-based species identification (4) from dried snake venom was used to solve a wildlife crime related to the illegal smuggling of venom in the state of Kerala in India.

Case History

The Judicial I Class Magistrate, Kannur Division, Kerala, forwarded three polythene packets containing dry crystals suspected to be snake venom to Laboratory for the Conservation of Endangered Species (LaCONES), Centre for Cellular and Molecular Biology (CCMB), Hyderabad, for species identification. The Kannur Flying Squad Range Officer and his staff confiscated these packets from a hotel room. Therefore, LaCONES had the critical task ahead to isolate DNA from the dried venom samples and then to establish conclusively the species of snake.

Materials and Methods

One-hundred milligrams of dried venom crystals were washed overnight with 500 μL of 1 × PBS (pH 7.2) and transferred to 1.5-mL Eppendorf tubes containing 500 μL of lysis buffer (100 mM NaCl, 50 mM Tris, 20 mM EDTA [pH 8.0]), 30 μL of 20% (w/v) SDS, 50 μL of DTT (500 mM stock), and 15 μL of proteinase-K (20 mg/mL stock) and incubated at 48°C for 4 h with gentle rotation in an hybridization oven. To reduce the inhibitory effect of proteins, 50 μL of DTT (500 mM stock) again was added to the lysis solution and incubated for another 4 h. After complete lysis of cells, genomic DNA was isolated using the conventional phenol-chloroform method (5). The DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and quantitated in 0.8% (w/v) agarose.

The DNA isolated from the venom was amplified using the universal primers mcb398 and mcb869 targeting the mitochondrial *cyt b* region (4). The amplification was carried out in 20 μL reaction volume containing 30 ng of template DNA, 10 mM each of dNTPs (Applied Biosystems Foster City, CA), 5 pmol of each primer (Bioserve, Hyderabad, India), 1.5 mM $MgCl_2$, 0.5 units of AmpliTaq Gold (Perkin-Elmer-Cetus, Norwalk, CT), and 1 × PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl). The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 35 cycles each of denaturation at 95°C for 30 sec, annealing at 52°C for 50 sec, and extension at 72°C for 1 min. The final extension was at 72°C for 10 min. The PCR products obtained were sequenced on both strands in triplicate, and the consensus sequence was analyzed using BLAST (6) program of National Centre for Biotechnology Information (NCBI).

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Results and Discussion

The cytochrome b sequence obtained from three dried venom samples (WL625, WL626, and WL627) showed maximum sequence similarity (99.0%) with the *cyt b* gene sequence of *N. naja* (NCBI Accession No. AF540932), that is, an Indian cobra. To conclusively settle the identity of the species, the DNA sequences obtained from the three dried venom samples were further aligned and compared with equal length (409 bp) of *cyt b* sequences of different Indian species of common venomous snake available in mitobase of NCBI, using MEGA 4.0 (7) program. Sequence comparisons identified 143 variable sites in total (Table 1), and pairwise comparisons differentiated all investigated species by a minimum of one nucleotide variation with a sequence similarity of more than 99.0% (Table 2). The other snake species showed <80.0% sequence similarity with the three dried venom samples under investigation. *Cyt b* gene is highly conserved region of mtDNA, and a very less intraspecific variation is observed in this region. Wuster and Thorpe (8) also have earlier shown an intraspecific variation of only 0.30% at the *CO I* (cytochrome oxidase I) gene in *Naja siamensis*.

On the basis of our report, the forwarding authority of the case filed a case in the court of law against the accused persons for illegal hunting of a highly endangered venomous snake, the Indian cobra, and for extracting and smuggling of snake venom. The decision is pending.

The universal primer-based molecular approach developed in our laboratory to establish the species identity has helped us in solving many wildlife-related crimes involving a variety of biological specimen (9–11). However, this is the first instance of DNA-based species identification from snake venom, following an assumption that because the venom might have been collected forcefully from the snake, there was a probability of the presence of epithelial cells in the venom from the salivary gland. This method would therefore greatly facilitate rapid forensic identification of snake species, thereby helping in reducing the volume of illegal trade of target species.

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TABLE 2—*Number of variable sites (above diagonal) and percentage similarity (below diagonal) among cytochrome b sequences from the Three venom samples (WL625, WL626, and WL627) and sequences of major indian venomous snake species.*

	WL625	WL626	WL627	*Naja naja*	*Echis carinatus*	*Bungarus caeruleus*	*Daboia russelii*
WL625	0	1	1	4	88	90	93
WL626	99.7	0	0	3	87	89	92
WL627	99.7	100.0	0	3	87	89	92
Naja naja	99.0	99.3	99.3	0	87	89	94
Echis carinatus	79.0	79.3	79.3	79.3	0	92	84
Bungarus caeruleus	78.6	78.8	78.8	78.8	78.1	0	89
Daboia russelii	77.1	77.4	77.4	76.9	79.3	78.1	0

TABLE 1—*Comparisons of cytochrome b sequences generated from venom samples with the sequences of major Indian venomous snake species available in National Centre for Biotechnology Information (NCBI) database.*

	Nucleotide Positions*							
				111111	1111111111	1111111111	1111111111	1111112222
Samples	111122	2233344555	6666777889	9999000000	0122222233	3334444445	5555566777	7888990111
	1347036912	5814703257	0147359281	4679013678	9513467901	2391234580	4678906125	8467695147
Naja naja	GGGGGTAGGA	GCTTCGATGT	ACGAGAACAA	GGCGGCTTGA	GTGGAGTGTA	GGCTAGAGAT	CGCACAAAGG	GGATGGTGGA
WL 625	....A.....	....	....	....	....	....	..T.......	....
WL 626	....A.....	....	....	....	....	....	..T.......	....
WL 627	....A.....	....	....	....	....	....	..T.......	....
Bungarus caeruleus	A.A..G.TAG	A.CATAG.TG	GGA....TG.	TATA.TG.AT	AGATGA..CG	A.TA.AT...	TAT.T.GT..	.TG.A.AAAG
Daboia russelii	TA.AAGGT.G	.TCGT..G.C	GG..A.GTGG	TTTT.T.ATC	A.AT...TGG	CTTATATTTA	T.GGAGG.AA	C....AGAAT
Echis carinatus	A..C.GGC.G	.....AGG.C	GG.GCTGTGG	TTTTATGGTC	AG..GAATA.	...GG.T.TG	G.GG......	...C.AA.TT
	2222222222	2222222222	2222222333	3333333333	3333333333	3333333333	334	
	1222233334	4455667778	8888999000	0111222333	4444456666	6777778899	990	
	9067957890	4729250170	1239258134	7039258147	0346921467	9136892814	793	
Naja naja	GAATGCCGTT	TTTGTGGTGG	ATATGAATGG	AATGGTGGAA	ACCCTATTGT	TGTCAGTTGA	GGA	
WL 625	....A.....	....	....	....	..T.......	....	...	
WL 626	....A.....	....	....	....	....	....	...	
WL 627	....A.....	....	....	....	....	....	...	
Bungarus caeruleus	.TG.TT..G.	GGGA..T.AA	GCGA.G..A.	.G.AAGAAGG	.T.G....A.	.T.TG...A.	TAT	
Daboia russelii	ATG.AT...G	GCGAAT..A.	.....GGA.A	GGGA....G.	GT.GC.G..G	A.CTGA..TT	...	
Echis carinatus	.GGCATTA..	AGG.AAAG.A	G..GAGGG..	.G.ACG.A.G	GG...G.C.G	..GAG.GA.G	...	

*Only variable sites shown. *Naja naja* is an Indian cobra, Accession No. AF540932; *Bungarus caeruleus* is a common krait, Accession No. AJ749305; *Daboia russelii* is Russel's viper, Accession No. AF471076; and *Echis carinatus* is an Indian saw-scaled viper, Accession No. GQ359433.

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