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DNA mini-barcoding: An approach for forensic identification of some endangered Indian snake species

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ABSTRACT

Illegal trade of snake skin and uncontrolled hunting have instigated the extermination of many endangered snake species. Efforts to check illegal trade are often impeded due to lack of proper species identification methods. Hence, conservation strategies demand for authentic and quick identification techniques to trace the origin of the seized samples. This study employs DNA mini-barcoding as a method to identify some endangered snake species of India. We have designed two sets of novel primers for targeting regions within the mitochondrial Cytochrome Oxidase I gene to produce 175 bp and 245 bp amplicons. 175 bp fragment was amplified in all 11 snake species studied while the 245 bp amplicon was obtained in 10 species. DNA mini-barcodes recovered from these amplicons enabled the identification of snake species by retrieving the sequences available in public databases. The similarity scores ranging from 98 to 100% (98% taken as threshold value for species identification) signify the consistency of these mini-barcodes in snake species identification. Moreover, the results of the validation study confirm the effectiveness of the technique in forensic perspective, where the diagnostic morphological features of the seized sample are often missing.

1. Introduction

Reptiles are widely hunted and traded for food, medicines, and other purposes in Southeast Asian countries [1]. Their demand with the lucrative profits has been a menace to the Indian wild fauna [2]. Illegal skin trade has threatened the survival of several snake species like the Indian Rock Python, Indian Rat Snake, Indian Cobra [3], and Red Boa [4]. Although, all these snake species are protected under the Indian Wildlife Protection Act, 1972, yet a clandestine trade continues undeterred. Hence, the conservation strategies require not only effective law enforcement but also robust techniques, for the identification of confiscated samples.

Morphological examination cannot be used for samples that have no visually recognizable features or the samples, which have been otherwise altered. In this regard, molecular identification techniques have proved to be an effective tool for species identification [5–12]. Among the advance molecular identification techniques, DNA barcoding technique using the 648 bp sequence of Cytochrome Oxidase I (COI) gene has proved to be a reliable tool for species identification [13–19]. This technique is based on the use of taxon specific primers for the amplification of a target region in the COI gene (usually from position 58 to 705 relative to mouse mitochondrial genome [20]) followed by sequencing the amplicon. The generated sequences are compared with the available reference sequences in the DNA databases to obtain species identification.

However, in the forensic scenario and in cases of formalin fixed museum samples, obtaining a 648 bp amplicon is often not feasible because of heavy DNA degradation [21,22]. Hence, DNA barcoding in such cases is not a realistic approach for species identification. Therefore, it is considered imperative to design a technique using novel primers to yield shorter amplicons, and thereby ensure augmented amplification probability. The sequences derived from these smaller amplicons are termed as 'mini-barcodes' and have been successfully employed in the identification of several animal species [23,24].

Hence, in this study we propose the use of mini-barcodes or short sequences using the novel primers designed herein for snake species identification. This is the first corroborative report on the application of mini-barcoding technique for the identification of some endangered snake species of India.

2. Materials and methods

2.1. Primer design

COI gene sequences of all available snake species were retrieved from GenBank and aligned using MEGA 3.1 software [25]. The conserved priming sites were identified in the alignment and primer set I was designed outside the established 648 bp barcode

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Table 1
Primers designed in the present study with their respective amplicon size.

Primer name	Sequence (5′–3′)	Amplicon size (base pairs)
Primer set I		
Minibar-F1	TGA TTY TTT GGH CAC CCR GAA GT	175
Minibar-R1	AAT ATR TGR TGG GCY CAD AC	
Primer set II		
Minibar-F2	GGT AGY GAT CAA ATC TTT AAY GT	245
Minibar-R2	GGG TAG ACD GTT CAV CCT GTT CC	

region, whereas the primer set II was designed so that the amplicon falls within the ambit of this region. The two sets of primers were carefully designed to yield smaller size amplicons (Table 1).

2.2. Sample collection and DNA extraction

The tissue and blood from the authenticated specimen of Indian Rock Python (*Python molurus*), Indian Cobra (*Naja naja*), Indian Rat Snake (*Ptyas mucosus*), Checkered Keelback (*Xenochrophis piscator*), Yellow Spotted Keelback (*Xenochrophis schnurrenbergeri*), Russell's Viper (*Daboia russelii*), Common Worm Snake (*Ramphotyphlops braminus*), Monocled Cobra (*Naja kouthia*), Red Boa (*Eryx johnii*) and Banded Krait (*Bungarus fasciatus*) were obtained from Snake Transit House, Jabalpur, Madhya Pradesh, India. Also, shed skin samples of Monocled Cobra (*Naja kouthia*) and Green Vine Snake (*Ahaetulla nasuta*) were obtained from Calcutta Snake Park, Kolkata, West Bengal, India. One formalin preserved sample of Banded Krait (*Bungarus fasciatus*) was provided by Zoological Survey of India (ZSI), Jabalpur, Madhya Pradesh, India. Genomic DNA from the tissue samples was isolated using QIAmp™ tissue DNA extraction kit (Qiagen, GmBH Germany) following the manufacturer's protocol, whereas the DNA from blood was isolated using standard Phenol Chloroform method [26]. The DNA from shed skin and formalin fixed tissue was isolated as per the protocols of Fetzner [27] and Shedlock [28], respectively. DNA samples were purified using Microcon-100 filter column (Millipore Corporation, MA).

2.3. PCR amplification and DNA sequencing

PCR was performed in 25 μl reaction volume containing 20 ng of DNA template, 5 mM $MgCl_2$, 1 mM dNTPs, 0.2 μM of each primer, 2.5 μl 10X buffer and 1.5 U of *Taq* polymerase (Invitrogen Life Technologies, Brazil) on GeneAmp® PCR system 9700 (Applied Biosystems). The PCR conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation (94 °C for 30 s), annealing (57 °C for 30 s for primer set I and 54 °C for 30 s for primer set II) and extension (72 °C for 30 s) with a final extension of 72 °C for 5 min followed by 4 °C hold. The amplified fragments were visualized on 2% agarose gel using ethidium bromide stain (0.5 μg/ml). The generated amplicons were cycle sequenced using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). DNA sequencing was performed on 3100 Avant Genetic Analyzer (Applied Biosystems).

Validation studies: The technique was validated with respect to the following points.

(i) *Reproducibility*: The reproducibility of the amplification of the targeted regions within COI gene was tested by performing the PCR in triplicates and comparing the sequences thus, obtained.
(ii) *Heteroplasmy*: DNA was extracted from different sources like tissue, blood and shed skin of a single individual and amplified to produce the targeted COI gene sequences, which were then compared.
(iii) *Stability*: The blood samples were deposited on filter paper, cotton cloth, metal, dried leaf and wood which were kept at ambient temperature for 10, 20 and 30 days.
(iv) *Sensitivity*: Effect of DNA concentration on PCR was tested using varied amounts of DNA templates, i.e., 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1 ng and 0.01 ng/μl.
(v) *Chemical treatments*: Effect of various chemicals on DNA recovery and subsequent PCR was tested by treating 25 mg of tissue samples with 0.1 M NaOH, 0.1 M HCl, 5% acetic acid, 5 M NaCl and 0.2% SDS in separate tubes, kept at ambient temperature for 5 days.
(vi) *Temperature treatments*: 25 mg of tissue samples were subjected to varying degrees of temperatures: (i) 37 °C for 10, 20, and 30 days, (ii) 56 °C for 12 h, 24 h and 36 h and (iii) 70 °C for 1 h, 2 h, 3 h and 5 h, separately.

Following the respective treatments, DNA was extracted and amplified in triplicates in a 25 μl reaction under the optimized conditions followed by sequencing of the products.
(vii) *Variation of PCR conditions*: (a) Concentration of $MgCl_2$ was varied from 1 mM to 10 mM. (b) Thermo-cycling parameters were changed from those optimized and mentioned above by varying annealing temperature ±3 °C and cycle number by ±2.

Positive and negative controls were included throughout.

2.4. Sequence analysis

All the sequences obtained were aligned using MEGA 3.1 software. In order to test the performance of the mini-barcodes in species identification, we calculated the variable and parsimony informative sites and the nucleotide divergences (using Kimura-2 parameter) between species in both the barcode datasets obtained from the two different primer sets, in MEGA 3.1 software. To identify the species, the sequences were subjected to similarity searches with the reference sequences in both the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BOLD systems (http://www.barcodinglife.org/).

3. Results

Expected sized PCR products, 175 bp for primer set I and 245 bp for primer set II were obtained for all the species studied (except, for *Ahaetulla nasuta*, which was not amplified with primer set II). Thus, the primer set I was 100% effective and the primer set II showed ~91% efficiency in amplifying the targeted region of COI gene from the samples used. Mini-barcodes were successfully recovered from all the 11 species studied, in case of primer set I and for 10 species in case of primer set II. We found 46% and 67% sites as variable and 29% and 28% sites as parsimony informative in the barcode data sets obtained from primer sets I and II, respectively. Mean sequence divergences were 21.9% and 33.4% for sequences derived from primer sets I and II, respectively (Table 2). The similarity searches in the public databases enabled identification

Table 2
Summary of genetic divergences (K2P model) at various taxonomic levels obtained using primer sets I and II.

Comparison within	Distances as obtained under K2P model		
	Min	Mean	Max
Primer set I			
Genus (among species)	0.0550	0.1382	0.2214
Family (among genera)	0.0547	0.2168	0.3608
Primer set II			
Genus (among species)	0.1072	0.1219	0.1367
Family (among genera)	0.0692	0.3346	0.9827