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Research Article

Preliminary genetic documentation of snake species through shed skin from Uttarakhand, India: A non-invasive genetic sampling approach

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Abstract

Non-invasive sampling is one of the most authentic techniques for the genetic study of endangered and rare animal species. In the present study based on non-invasive samples, we give the preliminary genetic documentation of snake species by using cytochrome *b* (Cyt *b*) and cytochrome c oxidase subunit I (COI) universal mitochondrial primers from Uttarakhand (UK), India. We sampled n=11 shed skin of unknown snake species from four different locations in Uttarakhand, India. The success rate of genomic DNA isolation, PCR amplification, and sequencing from collected samples was 100%. Afterward, in the genetics analysis, 8 out of 11 samples matched with Least Concern ver3.1 Rat snake species, two samples paired with Checkered keelback snake, and one sample matched with Indian cobra. Subsequently, 149 (Cyt b) and 207 (COI) species-specific fixed SNPs were observed. The obtained interspecific sequences divergences based on two mitochondrial loci among three snake species also show the high variability in the Uttarakhand snake population. The current study based on the non-invasive genetic sampling approach showed its importance in biodiversity conservation, especially those species which are under the endangered and critically endangered category. The genetic reference database of snake species helpful in species management, population, evolutionary-based study, and wildlife forensic in the future.

Keywords: Mitochondrial DNA and conservation, non-invasive genetic sampling, snakes, shed skin

Introduction

Non-invasive genetic sampling is a proportionately new approach for data-collection, has a great opportunity to explore the fauna in the wild. Through this approach, biologists can collect critical data of different wildlife animals without handling, capturing, or even observing individual animals (Mills et al., 2000; Carroll et al., 2018). Molecular techniques have proven to be crucial for identifying morphologically conservative species by extracting genetic material from hair, shed skin, feces, or other DNA source from non-invasive samples. The first time non-invasive genetic sampling was introduced as a method to obtain genetic samples from rare and elusive brown bears (*Ursus arctos*) in Europe in 1992 (Hoss et al., 1992; Taberlet & Bouvet, 1992) to study social structure in chimpanzees (*Pan troglodytes*) (Morin & Woodruff, 1992). According to a previous study, non-invasive samples do not require capture and handling of animals (Taberlet et al., 1999). This sampling has even greater value when the threatened and rare species are the objects of study. Today DNA can be successfully recovered from diverse animal sources such as hair, feathers, shed skins, etc. Non-invasive genetic sampling is used as an essential tool in the study of large carnivore species and reptiles species such as snakes because it is difficult to handle for sample collection. It used in the detection of rare species to forensic applications (Waits, 2004); conservation management of rare or cryptic species (Piggott & Taylor, 2003), population estimation (Boulanger et al., 2004; McKelvey & Schwartz 2010; Caroline et al., 2019), accurate data collection (Paetkau, 2003) and addressing genotyping errors (Bonin et al., 2004).

Nonetheless, snakes are a group of reptiles with many derived morphological characters related to their peculiar behavior and lifestyle. Snakes belong to the squamate reptiles, characterized by the regular shedding of the epidermis' outer layers, also known as ecdysis (Landmann, 1986; Maderson et al., 1998). In snakes, the epidermis' superficial layers are detached as a single, coherent sheet, whereas other Squamates (lizards and geckos) shed multiple smaller flakes. Most living reptiles are covered by scales of different sizes, thickness, variety, and color, and these characterize other species (Maderson, 1985a & b; Landmann, 1986; Alibardi, 2003 & 2006). Nowadays, mostly genetic studies based on high template DNA extracted from blood, liver, or other tissues. Unfortunately, it is challenging to draw blood from the veins of reptiles or birds, and its collection is hindered by the small body size of these species. As a result, reptiles often have to be killed for the acquisition of samples. Therefore, few molecular studies have been done on snakes' phylogeny, although non-invasive genetic sampling is an applicable method for such types of animals (Heise et al., 1995; Wong et al., 2004). Accordingly, we have standardized an efficient process for obtaining DNA from the cast-off skin of snakes instead of blood and amplify partial fragments of two mitochondrial genes, i.e., Cytochrome b (Cyt b) and Cytochrome c oxidase subunit I (COI). Through this method, we have obtained DNA for further genetic studies without killing the reptile. DNA-based techniques for species identification have recently started to be applied towards a wide variety of animals (Hryncewicz-Gwozdz et al., 2011; Kent et al., 2010) and proved an effective tool for reliable species identification phylogenetics and biodiversity analysis. They may be the key to successful work in law enforcement and conservation of wildlife (Dubey et al., 2011; Kumar et al., 2014; Rajpoot et al., 2017). DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify species (Hebert et al., 2003; Kress et al., 2005) and used to identify unknown species (Koch, 2010).

Uttarakhand is one of India's wealthiest hotspots, where a variety of floral, faunal, and microorganisms are present, including endangered, critically endeared, and rare animals and plant

species (Sundriyal & Sharma, 2016). However, several taxonomic and genetic study has been conducted on different reptiles; unfortunately, no single genetic research has been reported on snake species (Bahuguna, 2010; Rajpoot et al., 2016 & 2017). Therefore, the present study is the preliminary genetic documentation of snake species from Uttarakhand. Here, we apply the non-invasive approach and standardize a method to extracting DNA in high yield from snake cast-off skin; amplify a partial fragment of two mitochondrial genes i.e., 410bp Cytochrome b (Cyt b) and 658bp Cytochrome c oxidase subunit I; identify the species; provide authentic reference barcode database of snake species from Uttarakhand.

Material and methods

Sample Collection and Preparation for DNA Extraction from shed skin

Eleven shed skins of snakes were collected from four different districts (Dehradun, Haridwar, Rishikesh, and Dakpathhar) in Uttarakhand during the field Surveys conducted in 2017-2018 (Fig. 1 and Table 1). The shed skin exuviates for the entire snake were collected and washed with 70% Ethanol dried thoroughly and frozen until the extraction of DNA. Genomic DNA was extracted following the protocol of Fetzner (1999) with few modifications.

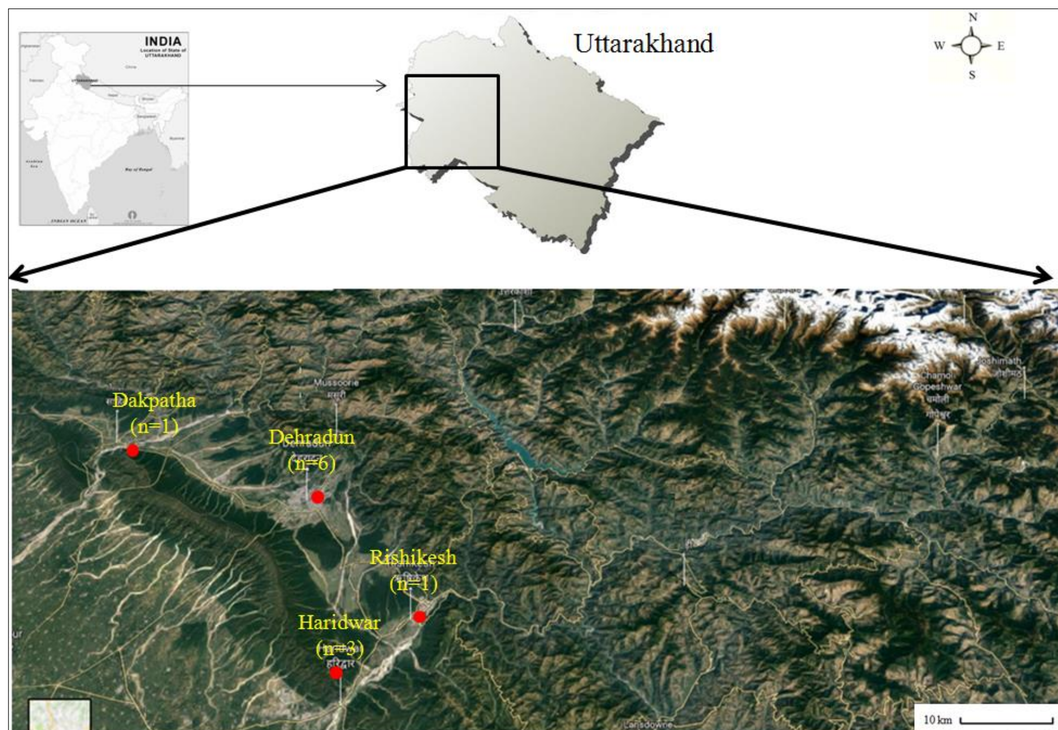

Figure 1. Map showing the location and number of collected shed Snake skins from four different places in Uttarakhand

Table 1. Details of used snake species from the present study and other sequences retrieved from Genbank. N indicates for number of samples used in present study; S: Sample; RS: Reference samples

Sample	Origin	N	Accession no. CYTB/COI	Conservation status
S-1, S-2, S-3, S-4, S-5, & S-6	Dehradun Near Narkonda village, Kaulagarh, ZSI campus	6	Present study	-
S-7	Rishikesh Near Ramjhula	1	Present study	-
S-8, S-9 & S-10	Haridwar near Motichur	3	Present study	-
S-11	Dakpathar near Assan barrage	1	Present study	-
Sequences retrieved from Genbank/References data				
***Ptyas mucosa* (PM) RS-PM1 & RS-PM2**	ZSI Collection/Genbank	2/1	LC105628.1/GQ225662.1	Least Concern ver3.1
***Xenophoris piscator* (XP) RS-XP1 & RS-XP2**	ZSI Collection/Genbank	2/1	FJ936561.1/ GQ225666	Not Evaluated
***Naja naja*(NN) RS-NN1 &RS-NN2**	ZSI Collection/Genbank	2/1	GQ225657.1/GQ225665.1	Not Evaluated

The approximately one-inch square piece was cut individually, chopped into small pieces with a surgical blade, and transferred to a sterile 2 ml Eppendorf tube from collected shed snake skins. After that, washed away with 10X phosphate-buffered saline (PBS) two times (kept for at least 20 min. on a shaking platform during each wash) and washed with nuclease-free water.

Shed skin (cut up) was placed into 300 µl of cell lysis buffer (10 mM Tris-base, 100 mM EDTA, and 2% sodium dodecyl sulfate (SDS, pH 8.0) along with 20 µl of Proteinase K (20 mg/ml). The samples were vortex thoroughly and then placed in a heat block or water bath at 56°C overnight. The samples are then removed from the heat block and cooled to room temperature (Note: the skin does not dissolve after Proteinase K digestion, but the DNA is released into the solution). The supernatant was transferred into a 1.5 ml Eppendorf tube and centrifuged at 8000 rpm for 3 min to pellet the remaining particles. The resultant supernatant was transferred to new tubes containing 600 µl 100% isopropanol, then mixed gently by inverting tubes, and then placed in a freezer –20°C for 12 hr. Extracts were then centrifuged at 16000 rpm for 20 min to pellet DNA, after which the isopropanol was poured off. Wash the pellet two times with 600 µl 70% EtOH (16 000 rpm for 1 min), after which the EtOH was poured off after each wash. Pelleted DNA was air-dried and then resuspended in 20µl TE (10 mM Tris, pH 8.0 + 1 M EDTA, pH 8.0). All centrifugation steps were performed in a cold centrifuge (4°C). The DNA concentration was quantified by absorbance measurement at 260nm. Absorbance measurements at 260nm were done using Cubimeter (Thermo Fisher Scientific Inc., Waltham, MA) with 4 µl of samples.

PCR Amplification and Data Analysis

Partial fragments of the Cyt b: 410bp (Verma et al., 2003) and COI: 658bp (Folmer et al., 1994) mitochondrial loci were successfully amplified. All PCR reactions were carried out on Eppendorf Master cycler (Orlando, USA) with a reaction set up of 10µl master mix volume, containing 4µl of 2X PCR master mix (Thermo Fisher Scientific), 0.5µl (0.025 mmol/L) each primer and approximately 1.5 to 2µl (45-50 ng) of genomic DNA and 3µl of ultrapure PCR grade water. Optimized PCR conditions of both mitochondrial loci as described in Rajpoot et al., (2016).

Thereafter, 3.5-4µl of PCR products were subjected to electrophoresis on 2% agarose (w/v) and visualized over the transilluminator to detect the amplification. Cautious positive and negative controls were incorporated throughout all DNA extraction and PCR amplification. Sanger sequencing is performed by a commercial using the same primers. All sequences obtained well for both the reverse and forward primers.
Qualities of sequences were determined using Sequence Analysis v5.2 software (Applied Biosystems, Foster City, CA). The Clustal W algorithm implemented in BioEdit version 7.0.5.3 (Hall, 1999) was used in the multiple sequence alignments (MSA). The sequences obtained from the three different species were compared with NCBI courses through a BLAST search (http://blast.ncbi.nlm.nih.gov/) and our reference data (Table 1). For the species diagnosis, we considered the percentage similarity between query and reference sequence. To confirm MSA results of both mitochondrial genes, compared with Neighbor-Joining (NJ) (Saitou & Nei, 1987) analysis conducted using MEGA version 7.0 (Kumar et al., 2016). Estimates of interspecific evolutionary divergence (d) over sequence pairs between groups determined by using the Kimura 2-parameter distance (Kimura, 1980) as implemented in MEGA version 7.0 (Kumar et al., 2016).

Results

The obtained genomic DNA concentration in all samples was good and ranged from 20.90 to 50.84 ng/µL. Interestingly, all shed skins, which are comparatively fresh or recently collected, yield slightly more quantity of DNA in comparison to old samples or earlier collected shed skins. In PCR amplification, Cyt b and COI mitochondrial loci were successfully amplified in all samples obtained readable sequences 410bp and 510bp, respectively. The obtained sequences of these two mitochondrial loci (Cyt b and COI) consisted of variables sites (128 and 185), Parsimony informative sites (126 and 179), and Singleton sites (2 and 6). Afterwards, the overall nucleotide compositions of the sequenced region of Cyt b was as follow: T=28.1%, C=28.9%, A=33.4% and G=9.6 %, while COI was as follow T=28.8%, C=27.0%, A=27.9% and G=16.3 %. Subsequently, in two mtDNA loci (Cyt b and COI) total of 149 and 207 fixed species-specific Single Nucleotide Polymorphic sites (SNPs) were observed among three snake species. The species wise details of nucleotide compositions and SNPs are given in Table 2 and Fig. 2.

Table 2. Nucleotide composition and species-specific fixed Single Nucleotide Polymorphic sites (SNPs) within two mitochondrial loci

Specimen	Len(nt) bp Cyt b COI	Nt composition Cyt b COI	SNPs Cyt b COI
Ptyas mucosa	410 510	T:28.0%,C:27.5%, A:35.9% & G:8.6% T:29.3%,C:26.4%, A:27.8% & G:16.5%	51 67
Xenophoris piscator	410 510	T:28.2%,C:31.0%, A:30.1% & G:10.6% T:28.5%,C:28.1%, A:28.5% & G:14.9%	42 76
Naja naja	410 510	T:28.2%,C:30.1%, A:30.6% & G:11.1% T:27.4%,C:27.8%, A:28.5% & G:14.9%	56 64

To validate our obtained query sample sequences of Cyt b and CO1 mitochondrial loci, they were compared with available Genbank sequences with the NCBI Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/BLAST). BLAST analysis of both mtDNA loci (Cyt b and

CO1) indicated that out of n=11, 8 samples (the S-1,S-3, S-5, S-7, S-8, S-9, S-10 and S-11) were highly matched (99%-100%) with the Indian rat snake (*Ptyas mucosa*), while two another samples (S-2 and S-4) were matched (99% and 100%) with Checkered keelback snake (*Xenophoris piscator*) and one sample S-6 was matched (100%) with Indian cobra (*Naja naja*) (Table 3).

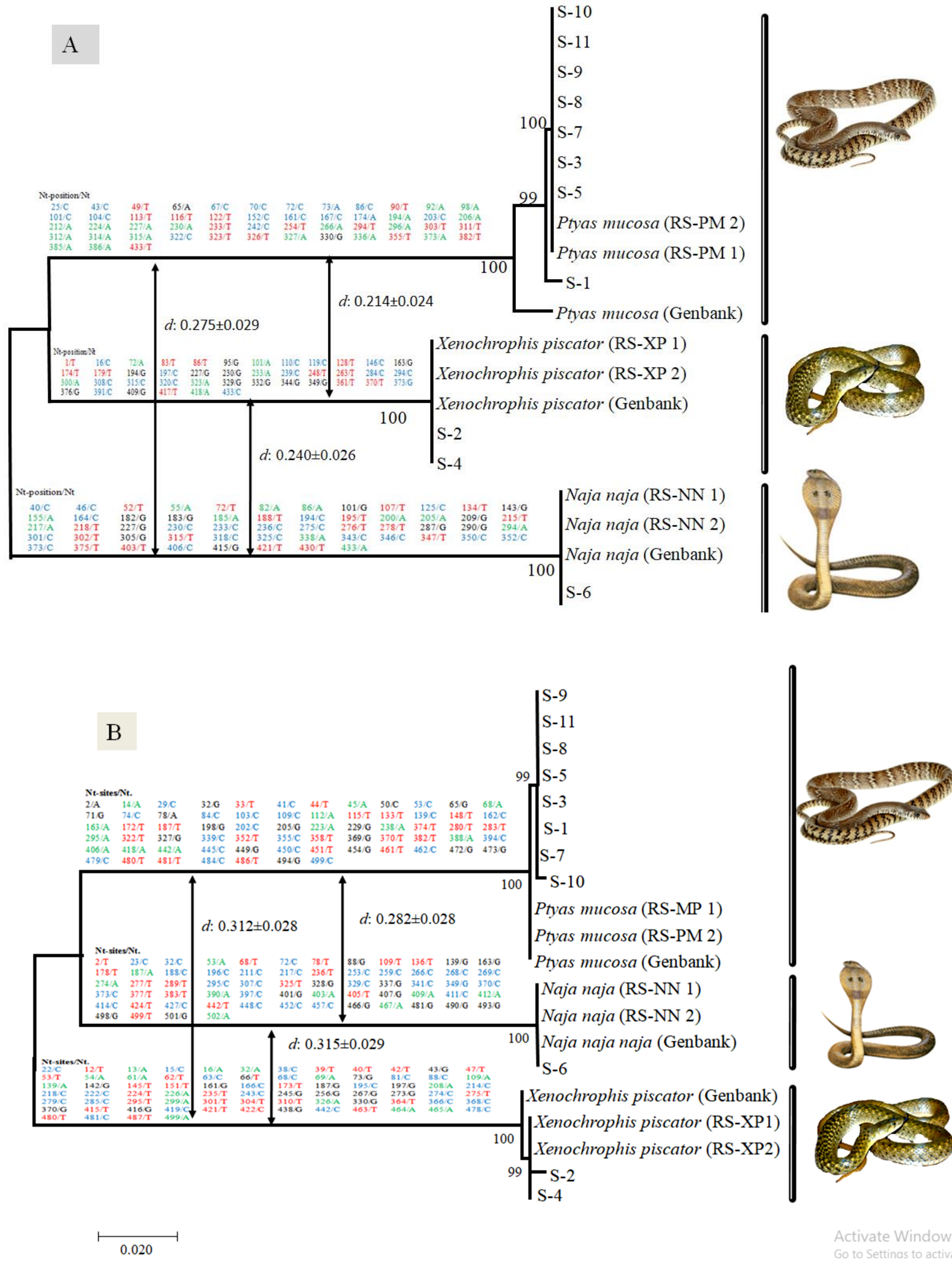

Figure 2. Cyt b (A) and COI (B) sequences based Neighbor-Joining (NJ) tree analysis to see the closet taxon with selected shed skin samples and references sequences of Snake. Above branch of both tree (A & B), the sequences and number represent the Cyt b and COI sequences based observed species-specific SNPs among three Snake species. After that, the arrow in both trees represents the Cyt b and COI sequences based on observed Interspecific sequence divergence (d) between three Snake species

To reconfirm our BLAST result, we matched obtained sequences of both mitochondrial loci with our reference sequences through MSA and all samples 100% matched with respective species correspondingly.

Table 3. Species identification result from the amplification genes and top similarity match (BLAST-reference sample) of used samples

Sample (S)	Species with the highest similarity COI (% BLAST Match)	Species with the highest similarity Cyt b (% BLAST Match)	Verified species
S-1	*Ptyas mucosa* (99-100)	*Ptyas mucosa* (100-100)	*Ptyas mucosa*
S-2	*Xenophoris piscator* (100-100)	*Xenophoris piscator* (100-100)	*Xenophoris piscator*
S-3	*Ptyas mucosa* (100-100)	*Ptyas mucosa* (100-100)	*Ptyas mucosa*
S-4	*Xenophoris piscator* (100-100)	*Xenophoris piscator* (100-100)	*Xenophoris piscator*
S-5	*Ptyas mucosa* (99-100)	*Ptyas mucosa* (100)	*Ptyas mucosa*
S-6	*Naja naja* (100-100)	*Naja naja* (100-100)	*Naja naja*
S-7	*Ptyas mucosa* (100-100)	*Ptyas mucosa*(100-100)	*Ptyas mucosa*
S-8	*Ptyas mucosa* (99-100)	*Ptyas mucosa* (99-100)	*Ptyas mucosa*
S-9	*Ptyas mucosa* (100-100)	*Ptyas mucosa* (100-100)	*Ptyas mucosa*
S-10	*Ptyas mucosa* (100-100)	*Ptyas mucosa* (100-100)	*Ptyas mucosa*
S-11	*Ptyas mucosa* (100-100)	*Ptyas mucosa* (100-100)	*Ptyas mucosa*

Thereafter, the NJ tree topology with download sequences from NCBI and our reference database also showed similar to BLAST result, where the unknown snake shed skins were clustered with to three species of snakes i.e., Indian rat snake (S-1, S-3, S-5, S-7, S-8, S-9, S-10, and S-11), Checkered keelback snake (S-2 and S-4) and Indian cobra (S-6) with a strong bootstrap value of 99%-100% (Fig. 2). Although the species tree topology of two mtDNA loci (Cyt b and COI) showed two different topologies, since in Cyt b the Checkered keelback snake present as a sister clade with Rat snake, while in COI, the Indian cobra present as a sister clade with Rat snake. Moreover, the obtained Cyt b and COI interspecific sequence divergence (d) between three snake species also support tree topology, wherein Cyt b the observed low interspecific divergence (d: 0.214±0.024) was from Rat snake to Checkered keelback snake and was high interspecific divergence (d: 0.275±0.029) from Rat snake to Indian cobra. Although, in COI sequence-based observed low interspecific divergence (d: 0.228±0.028) was from Rat snake to Indian cobra, while observed high interspecific divergence (d: 0.315±0.029) from Indian cobra to Checkered keelback snake (Fig.2).

Discussion

Out of eleven samples of shed skin, eight samples were found to be Rat snakes. It indicates a healthy population of Rat snakes in Uttarakhand. These cast-off skin samples were collected during our different field surveys, maximum skins were found near the agricultural area; it also indicates the increasing number of rat populations may increase the Rat snake population due to their feeding behavior. After observing the result, we will plan to conduct the population study of Rat snakes and determine the population demography of this snake species.

We advocate that shed skin is a precious object to study the evolutionary and population studies of snakes. Numerous scientific publications have documented the advances that non-invasive sampling has allowed in the field of molecular ecology over the past decades (Piggott & Taylor, 2003; Eggert et al., 2003). While some research also enlightens the flaws of non-invasive genetic sampling i.e., low success rate, a small quantity of DNA, etc. (Lobo et al., 2015).

However, in tree topology based on two mtDNA loci (Cyt b and COI), we observed the taxonomic relationship among the three species were different as the COI gene appears to possess a greater

range of phylogenetic signal than any other mtDNA regions (Hebert et al., 2003). Although most of the studies also showed the importance of Cyt b mtDNA loci in species separation in a short fragment of loci. In COI, the number of species fixed SNPs was more than of Cyt b, and it showed more variability within three snakes.

Conclusion

The present study unequivocally demonstrates the applicability of the non-invasive genetic sampling approach using Cyt b and COI mtDNA loci as a potential tool for the identification of snake species in India. Non-invasively collected samples reliably yielded sufficient DNA quality and quantity for genetic study; in fact, the current study is a conservative estimate of the value of shed snake skin for genetic studies as we intentionally targeted up to 500bp DNA fragments (658bp COI gene and 410bp Cyt b gene). A shed snake skin found in the field provides useful information regarding the population genetics and evolutionary studies. Further, a cast-off skin may heighten the monitoring programs' results by providing proof of a species presence, may also serve as voucher material without sacrificing an actual snake and without putting oneself in danger. More regular use of non-invasive genetic sampling would help advance the knowledge of species behavior, ecology, genetic, and evolutionary aspects of the snake and other species.

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