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Research Article

Genetic documentation of snake species using non-invasive sampling and non-toxic DNA isolation method

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Abstract

Despite the knowledge of the evolution of snakes worldwide, snake phylogeny requires a more detailed approach in South India. Molecular taxonomic approaches using DNA barcoding are a molecular tool frequently in species identification as well as studies of phylogenetics. Here, a non-invasive genetic sampling method using skin exuviates was used. This method is often overlooked for molecular studies of reptiles. We isolated DNA using a non-toxic method from skin exuviates collected from Chennai Snake Park and screened for the cytochrome oxidase subunit I (COI) region of mitochondria. Samples that were amplified successfully were barcoded. A total of seven species of snakes were identified which belonged to 5 families. We combined and compared sequences of these seven snake species from other countries to construct a phylogenetic tree and examined the genetic distance between species and families. This depiction and analysis showed a high degree of genetic variability intra-specifically between the South Indian samples to the samples from other parts of the world. This study documents how skin exuviates of snakes and the polymerase chain reaction of the COI region can be used for DNA barcoding and estimating phylogenetic relationships among snake species. Overall, this method is very versatile, inexpensive, and non-toxic which can help in understanding the evolution and phylogeny of snakes to formulate proper strategies for the conservation of snake species.

Keywords: Exuviates, phylogeny, reptilian, taxonomy, wildlife

Introduction

Snakes belong to the order Squamata and are further classified under the suborder Serpentes. Over 3500 species of snakes (Figueroa et al. 2016) have been recorded worldwide of which approximately 9% i.e., 270 species are found in India. Taxonomy based on morphological

characters for identifying snakes has limited power in resolving closely related snake species. This arises primarily because of the absence of the limbs and associated morphological variations (Kelly 2003; Khedkar et al. 2014). Furthermore, the phylogenies of snakes remain poorly known because the molecular studies show little similarity to each other or with morphological studies (Slowinski and Lawson 2002). Resolving the phylogenies finally helps biologists achieve a better understanding of the independent origins of various morphological characteristics, ecologies, and behavior of various taxonomic groups (Burbrink and Crother 2011).

Snakes belonging to higher groups are still under dispute since our understanding of phylogeny remains deficient. The evolutionary hypothesis could be tested using clade-wise species-level phylogeny of snakes (Figueroa et al. 2016). DNA Barcoding helps in identifying snake species as well as helps in assessing phylogeny. Evaluating DNA barcodes across geographic ranges is an effective tool for species identification (Laopichienpong et al. 2016). Other than resolving taxonomy ambiguities, DNA barcoding helps in resolving wildlife crimes such as the illegal trade of snakeskin and uncontrolled hunting using forensic investigation. Conservation strategies require authentic and quick identification techniques to trace the origin of the seized samples to identify some endangered snake species using DNA barcoding techniques. It can be useful to identify novel taxa, resolve taxonomic ambiguities, and monitor the illegal wildlife trade without the efforts of specialized experts (Nagy et al. 2012).

Non-invasive sampling is an innovative approach to data collection. Non-invasive sampling can also aid in the effective collection and critical data examination of different wildlife animals without handling, capturing, or even seeing individual animals (Mills et al. 2000; Carroll et al. 2018). Extraction of DNA from shed skin found in the field can be useful in identifying rare, elusive, or endangered species. Previous studies have shown that sufficient quantities of DNA can be obtained from shed skin which can be further used in a polymerase chain reaction (PCR) to identify varied snake species (Clark 1998). Reptilian breeding centers often decline requests for snake samples if the sampling requires harming their animals. Mostly, reptilian breeders are agreeable to giving away the shed skin which is discarded. Many methods have been proposed previously in quantifying the DNA obtained from shed skin. But such methods are labour-intensive or involve harmful or hazardous chemicals (Taggart et al. 1992).

The mitochondrial genome is maternally inherited and doesn't recombine, except for the control region all others are coding regions – hence no addition or deletion is expected and certain genes have a high mutation rate which helps in identifying species and subspecies. The ideal primer for targeting regions within the mitochondrial DNA to identify species is Cytochrome Oxidase I (COI) gene. COI shows a higher interspecific variation than the intraspecific variation between

animal taxa (Hebert et al. 2003; Chaves et al. 2008). The collection of DNA barcodes can help in the identification of endangered, rare, elusive species by recovering sequences available in public databases (Dubey et al. 2011) such as GeneBank and BOLD Systems. In this study, we attempted to illustrate a quick, inexpensive, and non-toxic method of extracting DNA from skin exuviates that can yield sufficient quantities of purified DNA for its use in PCR as well as for rapid identification methods for identifying snake species using DNA barcoding techniques. We compared the sequences generated to the sequences available from different countries in the GeneBank to estimate the genetic distance by demonstrating a viable use of skin exuviates in the taxonomic study.

Materials and methods

Collection of skin exuviates

Chennai Snake Park Trust (CSPT) is a captive center for endangered snakes. 40 shed skins of snakes were collected in a ziplock from captive enclosures of Chennai Snake Park, India. They were transported to the laboratory, kept dry, and stored at room temperature until DNA isolation.

DNA Extraction and PCR amplification

DNA extraction of the protocol was performed according to Fetzner, 1999 with slight modifications. The genomic DNA was visualized under 0.8% agarose gel electrophoresis. The presence of bands indicated the presence of genomic DNA. We used thermal cycler GX200 (Eppendorf) for PCR. COI universal marker was selected in this study. Samples were amplified with a final volume of 25 ml; containing 25-50 ng of genomic DNA, 1X PCR buffer optimized buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of forward and reverse primer, and 1.5 units of Taq DNA polymerase. Amplification condition was with the initial denaturation at 94^{o}C for 5 min followed by 25 cycles at 94^{o}C for 30s, 50^{o}C for 30 s, and 72^{o}C for 1 min, with an extension of 72^{o}C for 10 min. The resulting products that contained a clear band on gel electrophoresis were sequenced with ABI BigDye chemistry on an ABI 377 Genetic Analyzer.

Data analysis

Resulted sequences were checked and edited manually for miscalls and base spacing using the software BioEdit V7.0 (Hall, 1999). CLUSTALW was used for alignment (Thomson et al. 1997) and Sequences were submitted to GenBank. Obtained sequences were confirmed through NCBI BLAST (Basic Local Alignment Search Tool) (https: https://blast.ncbi.nlm.nih.gov). The intraspecific, interspecific, and intergeneric genetic divergences were calculated using MEGAX (Kumar et al. 2018). The NJ tree was edited in FigTree 1.4.2 (Rambaut 2012). The neighbor-

joining (NJ) tree was also checked to test the cluster pattern in the MEGA program. We downloaded sequences from NCBI after finding the species available in our sampling. We manually checked the data for base-calling errors, and used ClustalW multiple alignments in BioEdit to trim our sequences.

Results

A total of 40 skin exuviates were collected from Chennai snake park. DNA was isolated after rinsing it with PBS. Genomic DNA was visible in 35 samples. All samples were subject to PCR reactions irrespective of the presence or absence of genomic DNA. 27 samples that showed successful amplification was sent for Sanger Sequencing. Sequences were manually checked for sequencing quality, and base-calling errors and edited if necessary, using BioEdit. Eighteen sequences obtained fine quality which was used for further analysis. Obtained sequences were deposited in the GenBank. All the sequences were also confirmed through the BLAST and confirmed at the genus level and were placed in the same clade. A total of 7 genera and 7 species were identified belonging to 5 families respectively namely Colubridae, Elapidae, Viperidae, Boidae, and Pythonidae. The snakes identified were *Ptyas mucosa*, *Lycodon aulicus, Bungarus caeruleus, Malayopython reticulatus, Python molurus, Eryx johnii, Echis carinatus* using the COI gene. The sequences generated were submitted to GenBank (Table 1).

Table 1. List of taxa sequenced for this study with GenBank Accession Numbers

Family	Species	No. of samples	GenBank ID
Pythonidae	*Malayopython reticulatus*	1	MW144275
Colubride	*Ptyas mucosa*	7	MW144274 MZ827030- MZ827033, MZ379504, MZ379505
	Lycodon aulicus	1	MZ379511
Elapidae	*Bungarus caeruleus*	1	MZ827035
Pythonidae	*Python molurus*	6	MZ827036, MZ379506- MZ379510
Muraenidae	*Eryx johnii*	1	MW496346
Viperidae	*Echis carinatus*	1	OM818416
Sequences downloaded from NCBI			
Colubride	*Ptyas mucosa*	5	MW144274 KU529376 MH220714 LC105608 MK947911
	Lycodon aulicus	1	KT215868
Elapidae	*Bungarus multicinctus*	3	MN165154 JN860065 MN165157
Pythonidae	*Malayopython reticulatus*	4	MW144275 MF002008 KX012784 MH274436
	Python molurus	2	MH274580 AB920233
Viperidae	*Echis carinatus*	2	MG699966

			EU852307

Genetic Data Analysis

650 base pair amplicons were obtained which were trimmed to 600 base pairs using BioEdit software. A few sequences of identified snake species from different countries were downloaded from NCBI for comparison and grouped into their respective taxa using MEGAX Software. There were no COI sequences of *Bangarus caeruleus* and *Eryx johnii that* were available in GenBank, thus we used *Bungarus multicinctus*. No COI sequences of *Eryx johnii* was found thus only one sequence of this study was used for the analysis. Using DNASp we identified the number of polymorphic sites to be 229 with a total number of 356 mutations.

The genetic distances within the species were calculated using the Kimura-2 parameter. *Bungarus Sp.* showed a genetic distance of 0.10 due to differences at the species level. *Echis carinatus* and *Lycodon aulicus* observed a genetic distance of 0.05. *Ptyas mucosa, Python molurus,* and *Malayopython reticularis* showed a genetic distance of 0.01 (Table 2). The genetic distances within the families were calculated using the Kimura-2 parameter (Kimura, 1980). Elapidae calculated a genetic distance of 0.10 due to differences at the species level. Pythonidae exhibited a genetic distance of 0.09. Colubridae calculated a genetic distance of 0.06. While Viperidae showed a genetic distance of 0.05 with significant variations (Table 3).

Table 2. Genetic distances within snake species using Kimura 2 parameter

Snake Species	Genetic distances within species
Lepidodactylus lugubris	n/c
Ptyas mucosa	0.01
Echis carinatus	0.05
Bungarus Sp.	0.10
Python molurus	0.01
Lycodon aulicus	0.05
Malayopython reticulatus	0.01
Eryx johnii	n/c

Table 3. Genetic distances within snake families using Kimura 2 parameter

Snake Species	Genetic distances within families
Gekkonidae	n/c
Colubridae	0.06
Viperidae	0.05
Pythonidae	0.09
Elapidae	0.10
Boidae	n/c

Phylogenetic tree

MEGAX software was used for the construction of a phylogenetic tree using the Neighbour Joining phylogenetic tree with bootstrap value for 1000 replicates using a COI marker. Sequences obtained in this study were compared with sequences available in NCBI from different countries. Most genera or species were characterized in a separated clade of sequences obtained for this study or downloaded from the NCBI database. *Lepidodactylus lugubris* was taken as an outgroup to show the monophyletic clusters of snake species belonging to different families (Fig. 1).

The Colubridae family has two branches consisting of *Ptyas mucosa* and *Lycodon aulicus.* All the species showed as monophyletic cade and clustered within the families, namely the Elapidae, Pythonidae, Boidae, and Viperidae. *Bungarus caeruleus* and *Eryx johnii* having 650 bp sequences were generated for the first time. Since *Eryx johnii* COI sequences with 650 bp were not available in NCBI. Only the generated sequence was included (Fig. 1).

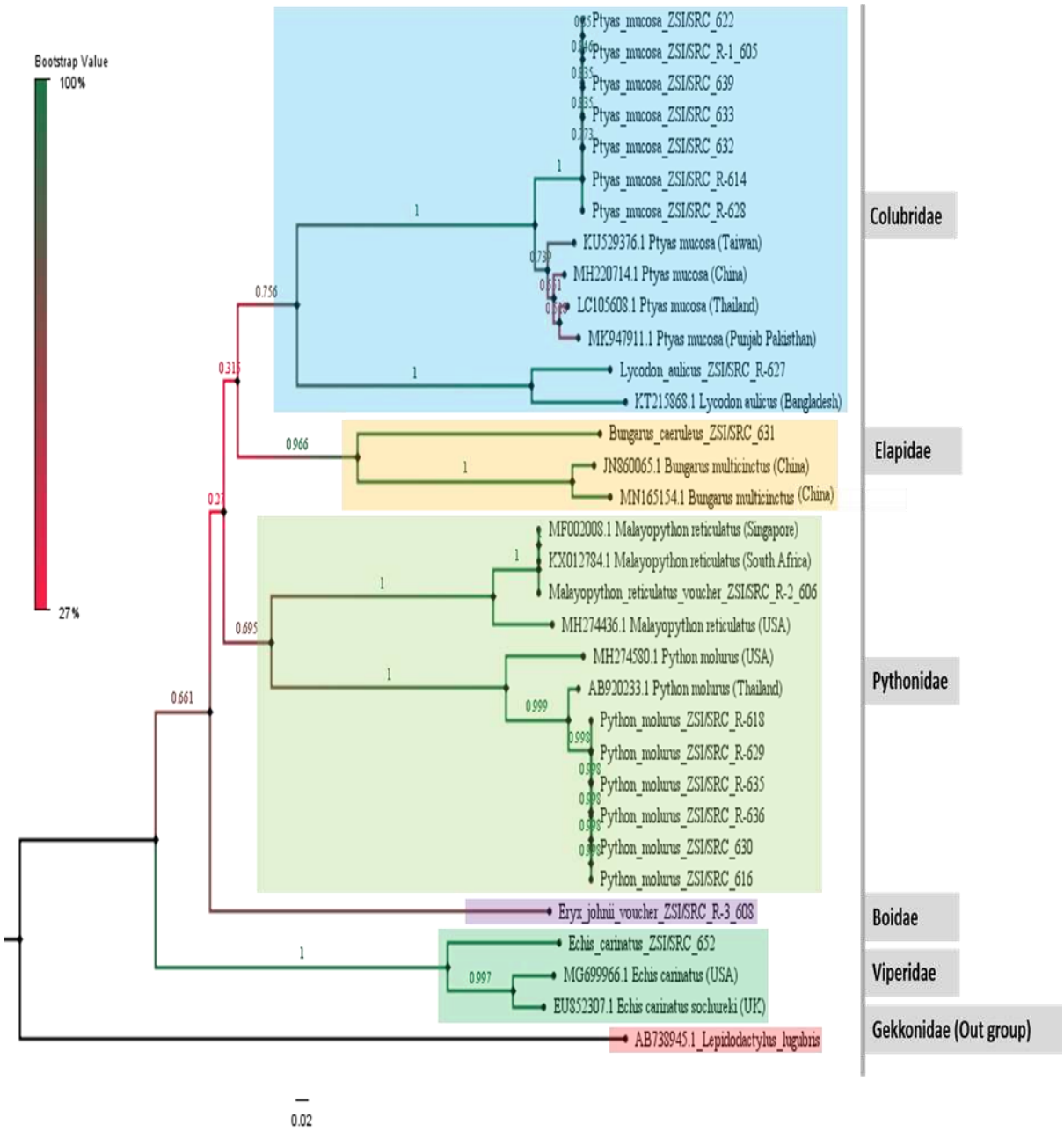

Figure 1. Phylogenetic tree using Neighbour-Joining (NJ) method for studied samples with NCBI data.

Discussion

Molecular taxonomic approaches using DNA barcoding could aid in snake identification and also use in studies of snake biodiversity. In this study 18 sequences were obtained from shed skins of snakes used, collected from captive enclosures of Chennai Snake Park. DNA was isolated with a nontoxic salt method (Fetzner 1999). Downloaded sequences were also combined with data. Obtained sequences were also confirmed through the BLAST and confirmed at the genus level and were placed in the same clade. A total of 7 genera and 7 species were identified belonging to 5 families respectively namely Colubridae, Elapidae, Viperidae, Boidae, and Pythonidae. Previous studies have shown the identification of unknown snakes using skin exuviates. In a

study in Maharashtra, 81 unknowns snake skin samples were validated efficaciously by DNA barcoding and compared with unknown samples for the assignment of taxonomic identity. A total of 23 species of snakes were identified, out of this, six species were listed under Endangered species (Red Data Book) (Khedkar et al. 2016). The similarity scores range from 98 to 100% in snake species identification (Dubey et al. 2011).

The genetic distances within the species were calculated using the Kimura-2 parameter. *Bungarus Sp.* showed a genetic distance of 0.10 due to differences at the species level. *Echis carinatus* and *Lycodon aulicus* showed a genetic distance of 0.05. *Ptyas mucosa, Python molurus*, and *Malayopython reticularis* showed a genetic distance of 0.01 (Table 2). The genetic distances within the families were calculated using the Kimura-2 parameter. Elapidae observed a genetic distance of 0.10 due to differences at the species level. Pythonidae exhibited a genetic distance of 0.09. Colubridae calculated a genetic distance of 0.06. Viperidae showed a genetic distance of 0.05 with significant variations (Table 3).

The phylogenetic tree was constructed using the Neighbour Joining method for 1000 replicates using a COI marker. Most genera or species were represented as a monophyletic clade. The sequences obtained in this study showed significant genetic variation from the sequences of other countries thus forming two separate branches within the same species. This variation was seen in almost all the snake families namely the Elapidae, Pythonidae, Boidae, and Viperidae. This might be due to subspecies or differences in ecology etc. The barcode sequences of *Bungarus caeruleus* and *Eryx johnii* were generated for the first time and submitted to NCBI. The COI phylogenetic tree supports the monophyly of studied snake genera with maximum bootstrap values with previous phylogenies (Nagy et al. 2012). Previous studies showed a 100% success rate in genomic DNA isolation, PCR amplification, and sequencing from collected shed skin samples (Rajpoot et al. 2021). The suspected cryptic diversity of snakes can also be identified using COI marker (Khedkar et al. 2016). The molecular-based examination of snakes also illustrates the existence of cryptic diversity in Indo-Bangladesh, China, and Thailand as verified in an earlier study (Ratnarathorn et al. 2019; Kundu et al. 2020).

Conclusion

This study documents how skin exuviates of snakes and the polymerase chain reaction of the COI region can be used for DNA barcoding and estimating phylogenetic relationships among snake species. Snake shed skin can be used as a resourceful material for genetic studies instead of snake tissue. Overall, this method is very versatile, inexpensive, and non-toxic which can help in understanding the evolution and phylogeny of snakes to formulate proper strategies for the conservation of snake species.

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