***Short Research Article***

# Molecular Identification of Captive Snakes from their Shed Skin Using Cyt *b* & 12s rRNA Markers


## ABSTRACT

**Aims:** To successfully utilize non-invasive shed skin samples for species identification of captive snake species of Tamil Nadu and create genetic repository.
**Study design:** The experiment was designed to apply the ammonium acetate method of DNA extraction from shed skin. Two mitochondrial markers were used to ascertain identification of species.
**Place and Duration of Study:** Advanced Institute for Wildlife Conservation (AIWC), Tamil Nadu Forest Department, Vandalur, Chennai, Tamil Nadu. The samples were collected between May and October 2019 from Arignar Anna Zoological Park, Vandalur, Guindy National Park, Chennai, and Amirthi Zoological Park, Vellore, Tamil Nadu.
**Methodology:** We collected fresh shed skin from 8 different snake species from captivities of zoos and dried between 48 to 72 hours to remove moisture. Independent DNA isolations were performed for each sample. The DNA isolated samples were quantified using Nanodrop Spectrophotometer for concentration. Independent PCR amplification of mitochondrial regions of cytochrome b and 12S rRNA were performed and agarose gel electrophoresis was carried out. PCR products were subjected to sanger sequencing using genetic analyzer.
**Results:** The DNA concentration from all 8 different snake species ranged between 250 to 1600 ng/µL and average quality ratios $A_{260/280}$ of 1.85 and $A_{260/230}$ of 2.10. Both the mitochondrial gene regions cytochrome b and 12S rRNA showed specificity in species amplification with NCBI BLAST result ranging from 99-100%. Phylogenetic trees using maximum-likelihood method classified closely related species under the same clade, with a bootstrap support of 60-100%. Genetic distances of snake species ranged from 0.148-0.457 in cytochrome b region and 0.148-0.457 in 12S region.
**Conclusion:** Shed skin is often overlooked from utilization for species identification. In this study, DNA from shed skin of 8 captive snakes is extracted and amplified using Cyt *b* and 12s mitochondrial markers. Individual phylogenetic trees are constructed for each marker to find relatedness of different snake species with one another. This work is an initiation of genetic repository creation of captive snake species of Tamil Nadu and could be effectively employed in conservation and population genetic studies of snakes.



*Key words: Skin Exuviates, mtDNA markers, Molecular Phylogeny, Wildlife Protection Act*


## 1. INTRODUCTION

The application of molecular genetics has become an indispensable part of reptilian taxonomy and systematics, population genetics, ecosystem and disease management, conservation, and wildlife forensic investigation in recent decades. While it has proven to be extremely utilitarian, the down side of this is the sample requirement to carry out molecular assays that are often collected by invasive methods such as drawing blood from caudal vein or cardiac puncture or even sacrifice of the

animal [1]. Snakes and other reptiles, in general, have an advantage over other vertebrates in that they shed their skin periodically in a process called ecdysis. This process is more pronounced in snakes, as the old epidermal layer is completely sloughed off from the body as a sheet of keratin [2]. The use of such cast off skin as source of DNA was recognized by the Knight (1992) [3], following which Bricker (1996) and Clark (1998) [4] modified existing DNA extraction protocol for pure and high yielding method from reptile shed skin. However, it was Fetzner's protocol [5] with ammonium acetate proved to be an easy and efficient technique to extract DNA from reptile shed skin.

India is home to about 344 species of snakes, whose numbers keep growing due to new discoveries every year. Every new discovery is validated by unique genetic sequence of the species so as to avoid misidentification by traditional morphological method. In addition to species identification molecular genetics of a species is essential for conservation and management strategies [6]. Using shed skin of snakes, researchers were not only able to obtain good quality and quantity of DNA, but have amplified mitochondrial genes like *16s* rRNA gene [7]; mini-barcodes using Cyt *b* gene (175bp - 245 bp) [8]; Cyt *b* gene (~431 bp) using PCR-RFLP method [9]; CO I gene (~652 bp) for 23 species of snakes [10]; Cyt *b* gene (401 bp) and COI gene (~658 bp) [11] in the Indian scenario.

In our present study, we have successfully isolated DNA and amplified two mitochondrial genes, Cyt *b* and 12s rRNA from eight common snake species by PCR technique for the identification of species from shed skin. Through this, we aim to create a reference genetic database to effectively serve future applications in study of phylogeny, biodiversity, wildlife forensics, significant conservation measures and corroborations of unknown snake species.

## 2. MATERIALS AND METHODS

### 2.1. Sample Collection

Exuviates from Indian Rock python (*Python molurus*) (n=9), Reticulated python (*Malayopython reticulatus*) (n=2), Indian Cobra (*Naja naja*) (n=7), Russell's viper (*Daboia russelii*) (n=4), Red Sand Boa (*Eryx johnii*) (n=1), Long nosed vine snake (*Ahaetulla oxyrhyncha*) (n=2), Common Krait (*Bungarus caeruleus*) (n=2), and King Cobra (*Ophiophagus hannah*) (n=1) were collected between May and October 2019 from Arignar Anna Zoological Park, Guindy National Park, Chennai, and Vellore Amirthi Zoological Park, Tamil Nadu. Samples were collected from individual cages, dried for 48-72 hrs to remove moisture by spreading the shed skin on a blotting paper at room temperature, and stored at room temperature in airtight zip lock bags [5]. The snake skin exuviates used in this study were those stored for more than a year.

### 2.2. DNA Extraction and PCR

Approximately 1 square inch of skin exuviate was homogenized in lysis buffer (10 mM Tris-base, 10 mM EDTA, 2% SDS, pH 8.0 and 0.2 mg/mL Proteinase K) at 56 °C for 4-6 hrs. DNA was isolated using ammonium acetate followed by isopropanol precipitation as described by Fetzner (1999) [5]. Extracted DNA was resuspended in nuclease-free water and stored at (-20 °C). Partial fragment of Cyt *b* and 12s rRNA genes were amplified using universal primers [12]. Independent PCR for both the target genes was carried out in Eppendorf Nexus GSX1 Mastercycler. The reaction tubes of 10 µL total volume were set up containing 1X Taq Buffer (KAPA Biosystems, SIGMA), 0.25 mM dNTPs, 0.4 µM of both forward and reverse primer, 2.5 mM $MgCl_2$, 0.25 U Taq DNA Polymerase (KAPA Biosystems, SIGMA) and 1 µL of template DNA. Cycling conditions consisted of 5 min. of initial denaturation at 95 ˚C, followed by 35 cycles of 30 seconds of denaturation at 95 ˚C, 30 seconds of annealing at 57 ˚C, 45 seconds of extension at 72 ˚C and final extension at 72 ˚C for 10 min. The PCR reactions were set with positive and non-template controls. The PCR amplicons obtained were visualized by electrophoresis on 2% agarose gel using BioRad XR+ Gel documentation system.

### 2.3. DNA Sequencing and Bioinformatics Approach

The PCR products were purified using QIAquick gel extraction kit (Qiagen, Germany) and sequenced bi-directionally by Sanger sequencing in ABI 3730 DNA Analyzer (Applied Biosystems, USA) using ABI Big Dye TM Terminator Cycle sequencing kit (Applied Biosystems, USA) [8]. The forward and reverse sequences of each sample were aligned and trimmed at both ends and assembled using MEGA X software and the sequences were searched against the GenBank database using BLASTn.

The phylogenetic tree was constructed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms software [13] using Maximum Likelihood tree using Kimura 2-parameter model was reconstructed with 500 bootstrap replicates for Cyt b and 12s rRNA genes.

## 3. RESULTS AND DISCUSSION

The DNA isolated from the skin exuviate samples were of good quality with concentration ranging from 250 to 1600 ng/µL and average quality ratios $A_{260/280}$ of 1.85 and $A_{260/230}$ of 2.10. Partial fragments of Cyt *b* and 12s *rRNA* genes were amplified in a total of eight snake species. Sequences obtained were matched against NCBI using BLAST tool. Percentage similarity using BLAST ranged from 99-100% thereby demonstrating the versatility and discriminatory power of the primers in amplification of snake species used. Table.1 displays the Accession number of sequences submitted to the NCBI database.

**Table 1. Base pair length and accession numbers of sampled snake species submitted to NCBI GenBank.**

| S.No. | Species | Cyt *b* | | 12s rRNA | |
|---|---|---|---|---|---|
| | | Base pair length | GenBank accession number | Base pair length | GenBank accession number |
| 1 | Indian rock python | 359 bp | MZ331840 | 297 bp | MW882912 |
| 2 | Reticulated python | 291 bp | MW684177 | 415 bp | MZ021338 |
| 3 | Red sand boa | 334 bp | MZ029430 | 390 bp | MZ407592 |
| 4 | Common vine snake | 337 bp | MZ029428 | 426 bp | MZ028097 |
| 5 | Common Krait | 353 bp | MZ052222 | 413 bp | MZ333471 |
| 6 | Indian Cobra | 317 bp | MZ029431 | 398 bp | MZ333472 |
| 7 | King Cobra | 364 bp | MZ029429 | 369 bp | MZ028096 |
| 8 | Russel's Viper | 298 bp | MZ029432 | 367 bp | MZ028090 |

Phylogenetic tree reconstruction using maximum-likelihood method classified closely related species under the same clade, with a bootstrap support of 60-100% (Figure. 1a &1b). Pair-wise genetic distance matrices were constructed using Tamura-Nei model with gamma distribution (Table 2). Minimum interspecific genetic distance among Cyt *b* sequences was observed between *P. molurus* and *E. johnii* while maximum interspecific distance was between *B. caeruleus* and *P. reticulatus.* The interspecific genetic distances ranged from 0.148 to 0.457 (average 0.286).

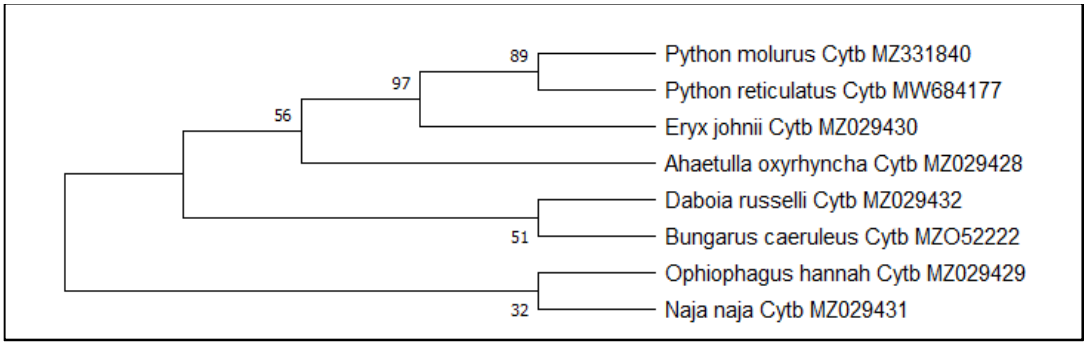


**Fig.1 (a). Phylogenetic Neighbour-joining tree reconstruction using Maximum Likelihood method and Kimura 2-parameter model with 500 bootstrap replicates for Cyt *b* gene**

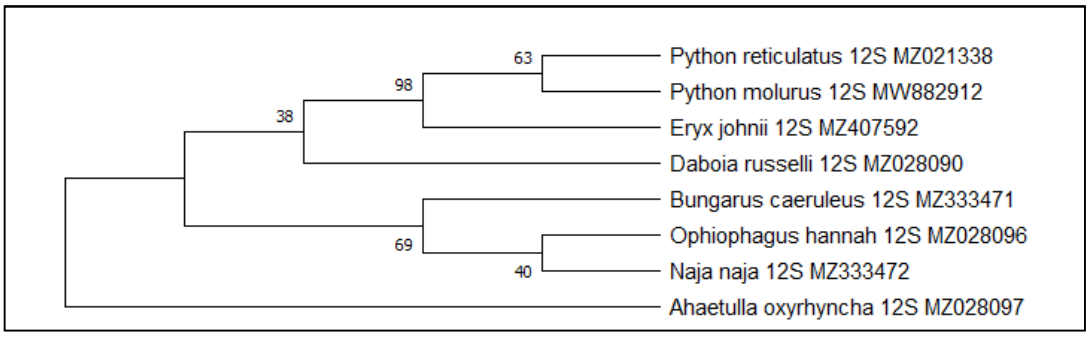


**Fig.1 (b). Phylogenetic Neighbour-joining tree reconstruction using Maximum Likelihood method and Kimura 2-parameter model with 500 bootstrap replicates for 12s rRNA gene.**

**Table 2. Genetic distances of 8 snake species based on mtDNA genes Cyt *b* and 12s rRNA**

| Amplified mtDNA gene | Interspecific distance | Average interspecific distance |
|---|---|---|
| **Cytochrome b** | 0.148-0.457 | 0.286 |
| **12s rRNA** | 0.078-0.322 | 0.203 |

Analysis of 12s rRNA sequences yielded minimum interspecific genetic distance between *P. molurus* and *P. reticulatus* and maximum interspecific distance between *D. russelli* and *E. johnii*, with the genetic distances ranging from 0.078 to 0.322 (average 0.203). Table 2 shows the pattern of existing relationships among the selected snake species.

The phylogenetic trees from Cyt *b* and 12s rRNA genes shows relatedness of snakes according to the earlier taxonomic classifications [23]. Among the eight species, the Indian Rock Python and Reticulated python are protected under Schedule I of the Indian Wildlife (Protection) Act (WPA), 1972 which is the highest level of protection for wild animals. Although the Indian cobra (*N. naja*), King cobra (*O. hannah*), Russel's viper (*D. russelii*) is placed in Schedule II of WPA and the rest in schedule IV, it still invites legal punishment if the snakes are harmed (Table 3). Hence, conservationists and researchers can opt for snake skin exuviates to carry out arduous research in snakes like sexing of individuals [14] for captive breeding in zoological parks and studying the phylogenetic relationship between species. Another advantage of using shed skin is that it does not require any preservation methods like ethanol treatment [10] or deep freezing [1,11,14,15] prior DNA isolation and PCR processes.

**Table 3. Legal protection statuses of snake species**

| S.No. | Species | Family | Wildlife Protection Act status | IUCN status |
|---|---|---|---|---|
| 1 | Indian rock python (*Python molurus*) | Pythonidae | Schedule I (Part II) | Near Threatened [16] |
| 2 | Reticulated python (*Malayopython reticulatus*) | Pythonidae | Schedule I (Part II) | Least Concerned [17] |
| 3 | Red sand boa (*Eryx johnii*) | Boidae | Schedule IV | Near Threatened [18] |
| 4 | Common vine snake (*Ahaetulla oxyrhyncha*) | Colubridae | Schedule IV | Not listed |
| 5 | Common Krait (*Bungarus caeruleus*) | Elapidae | Schedule IV | Least Concerned [19] |
| 6 | Indian Cobra (*Naja naja*) | Elapidae | Schedule II (Part II) | Least Concerned [20] |
| 7 | King Cobra (*Ophiophagus hannah*) | Elapidae | Schedule II (Part II) | Vulnerable [21] |
| 8 | Russel's Viper (*Daboia russelii*) | Viperidae | Schedule II (Part II) | Least Concerned [22] |

## 4. CONCLUSION

The universal primer based molecular approach on non-invasive snake skin exuviates has benefitted us in studying the phylogenetic relationship of different snake species and contributed to the primary creation of robust genetic reference library. This research has also yielded new genetic sequences of 12s rRNA genes of 450-500 bp length which can be used as an alternative gene to identify snake species of India. The study yielded for the first time, the genetic sequence of the common krait (*Bungarus caeruleus*) from south India which can be utilitarian in studying genetic variation within species of venomous snakes. This work could facilitate the implementation of conservation measures and wildlife crime examinations using mitochondrial markers non-invasively. The nonexistent genetic information of other species of snakes can be added following above said method without causing any harm to the creatures.

## REFERENCES


1. Bricker J, Bushar LM, Reinert HK, Gelbert L. Purification of high-quality DNA from shed skins. Herpetological Review. 1996; 27(3): 133-133.

2. Lillywhite, HB, Sheehy, CM. Synchrony of ecdysis in snakes. Herpetol Conserv Biol. 2016; 11(2): 286-292.

3. Knight, RA. Molecular systematics of the Agkistrodon complex (Doctoral dissertation, Texas Tech University). 1991.

4. Clark A. Reptile sheds yield high quality DNA. Herpetological Review. 1998; 29(1): 17-17.

5.Fetzner Jr JW. Extracting high-quality DNA from shed reptile skins: a simplified method. Biotechniques. 1999; 26(6): 1052-1054.

6. Horreo JL, Peláez ML, Fitze PS. Skin sheds as a useful DNA source for lizard conservation. Phyllomedusa, 2015; 14(1): 73-77.

7. Dubey B, Meganathan, PR, Haque I. Multiplex PCR assay for rapid identification of three endangered snake species of India. Conservation Genetics. 2009; 10(6): 1861-1864.

8. Dubey B, Meganathan PR, Haque I. DNA mini-barcoding: an approach for forensic identification of some endangered Indian snake species. Forensic Science International: Genetics, 2011; 5(3): 181-184.

9. Dubey B, Meganathan PR, Haque I. Molecular identification of three Indian snake species using simple PCR–RFLP method. Journal of forensic sciences. 2010; 55(4): 1065-1067.

10. Khedkar T, Sharma R, Tiknaik A, Khedkar G, Naikwade BS, Ron TB, Haymer D. DNA barcoding using skin exuviates can improve identification and biodiversity studies of snakes. Mitochondrial DNA Part A. 2016; 27(1): 499-507.

11. Rajpoot A, Kumar VP, Bahuguna A, Rasaily SS. Preliminary genetic documentation of snake species through shed skin from Uttarakhand, India: A non-invasive genetic sampling approach. Journal of Wildlife and Biodiversity. 2021; 5(1): 81-91.

12. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences. 1989; 86(16): 6196-6200.

13. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution, 2018; 35(6): 1547.

14. Tawichasri P, Laopichienpong N, Chanhome L, Phatcharakullawarawat R, Singchat W, Koomgun T, Srikulnath K. Using blood and non-invasive shed skin samples to identify sex of caenophidian snakes based on multiplex PCR assay. Zoologischer Anzeiger. 2017; 271: 6-14.

15. Jadhav AS. DNA barcoding of Python molurus using cytochrome b gene sequences analysis. DNA, 2017; 2(5): 188-191.

16. Aengals A, Das A, Mohapatra P, Srinivasulu C, Srinivasulu B, Shankar G, Murthy BHC. *Python molurus*. The IUCN Red List of Threatened Species. 2021: e.T58894358A1945283. https://dx.doi.org/10.2305/IUCN.UK.2021-2.RLTS.T58894358A1945283.en. Accessed on 29 July 2022.

17. Stuart B, Thy N, Chan-Ard T, Nguyen TQ, Grismer L, Auliya M, Das I, Wogan G. *Python reticulatus*. The IUCN Red List of Threatened Species. 2018: e.T183151A1730027. https://dx.doi.org/10.2305/IUCN.UK.2018-2.RLTS.T183151A1730027.en. Accessed on 29 July 2022.

18. Anderson S, Papenfuss T, Srinivasulu C, Kulkarni NU, Mohapatra P, Milto K, Bhattarai S, Vyas R, Ganesh SR, Thakur S. *Eryx johnii*. The IUCN Red List of Threatened Species. 2021: e.T164733A1071806. https://dx.doi.org/10.2305/IUCN.UK.2021-2.RLTS.T164733A1071806.en. Accessed on 29 July 2022.

19. Mohapatra P, Giri V, Suraj M, Das A, Srinivasulu C, Kandambi D, Ukuwela K. *Bungarus caeruleus*. The IUCN Red List of Threatened Species. 2021: e.T172702A1369896. https://dx.doi.org/10.2305/IUCN.UK.2021-3.RLTS.T172702A1369896.en. Accessed on 29 July 2022.

20. De Silva, A, Ukuwela K, Shankar G, Srinivasulu B, Das A, Vyas R, Sawant NS, Kulkarni NU, Deepak V, Thakur S, Mohapatra P, Srinivasulu C, Achyuthan NS. *Naja naja*. The IUCN Red List of Threatened Species 2021: e.T62241A3110222. https://dx.doi.org/10.2305/IUCN.UK.2021-3.RLTS.T62241A3110222.en. Accessed on 29 July 2022.

21. Stuart B, Wogan G, Grismer L, Auliya M, Inger RF, Lilley R, Chan-Ard T, Thy N, Nguyen TQ, Srinivasulu C, Jelić D. *Ophiophagus hannah*. The IUCN Red List of Threatened Species. 2012: e.T177540A1491874. https://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T177540A1491874.en. Accessed on 29 July 2022.

22. Suraj M, Narayanan S, Srinivasulu C, Mohapatra P, Giri V, Murthy BHC, de Silva A, Ukuwela K, Kannishka S. *Daboia russelii*. The IUCN Red List of Threatened Species. 2021: e.T172710A1372376. https://dx.doi.org/10.2305/IUCN.UK.2021-3.RLTS.T172710A1372376.en. Accessed on 29 July 2022.

23. Uetz, P., Freed, P, Aguilar, R. & Hošek, J. (eds.) (2022). The Reptile Database, http://www.reptile-database.org.