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Snake Phylogenetic Analysis Of Cytochrome B Gene Sequence

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Abstract

Phylogenetic analysis of two species of snake *Eryxcolubrinus loveridgei* and *Ptyas mucosus* was investigated using the gene encoding cytochrome b. The nucleotide sequences of complete and partial mtDNA cytochrome b were determined in numerous specimens. Sequence divergence between species and genera was evenly distributed in the cytochrome b gene but rather high compared to reports for other fish species. Phylogenetic analyses on complete cytochrome b were used to study the relationships among the considered species. The molecular phylogeny of sample was determined by analyzing cytochrome b gene sequences. On the basis of position of sequence of the given python sample in the phylogenetic tree, the sample showed closest similarity with *E. loveridgei* and *P. mucosus.*

Keywords: *Eryxcolubrinus loveridgei*, *Ptyasmucosus*, DNA sequence, Cytochrome b, Phylogenetic analysis.

INTRODUCTION

DNA barcoding is a simple technique used to develop a large-scale system of classification that is broadly applicable across a wide variety of taxa. Molecular phylogenetics is the analysis of hereditary molecular differences, mainly in DNA sequences, to gain information on an organism's evolutionary relationships. Molecular phylogeny of snake was determined by analyzing cytochrome b gene. Phylogenies form the cornerstone of our understanding of evolutionary relationships between organisms and provide a historical basis for testing and inferring ecological and evolutionary processes. (Rieppel, 1988) provided useful historical reviews of progress in understanding snake phylogeny and classification. Hydrophiine or 'true' sea snakes are a diverse radiation of fully marine venomous species that belong to the same evolutionary lineage as venomous terrestrial elapids.

Snakes represent a taxonomically underdeveloped group of animals in India with a lack of experts and incomplete taxonomic descriptions being the main deterrents to advances in this area. Veterinary and forensic science laboratories frequently encounter samples lacking any morphological details that make it impossible to identify them as meat, leather, bones, blood stains on clothes. Therefore, there is a need to determine the origin of anonymous biological traces. In addition, species identification represents a key aspect of biodiversity studies (Ardura *et al*., 2011). The molecular markers and DNA sequencing have been taken as good markers to classify the taxonomy and phylogenetic relationships among species. The application of PCR technique has significantly improved the efficiency of laboratorial diagnostic procedures by allowing the in vitro amplification of a large number of DNA copies using a specific genomic region as template, followed by complementary techniques (Fajardo *et al*., 2007). Since it only requires a small amount of template DNA, the PCR method has been particularly useful for the identification of species in suboptimal DNA samples like forensic samples and blood stains, also in archaeological remains and museum specimens owing to the highly degraded and fragmented nature of ancient DNA secular and morphological data sets will ultimately be necessary to develop a comprehensive phylogeny of snakes and each data source can make a unique contribution. On one hand, molecular methods can provide large quantities of phylogenetically informative

data. Although data have been plentiful, colubroid molecular phylogenies have been unstable due to their inherent sensitivity to taxon sampling (Kelly *et al*., 2003; Kraus & Brown, 1998). On the other hand, only few morphological complexes have been analyzed thoroughly within snakes, and the paucity of broadly sampled morphological characters has prevented the compilation of a large morphological data matrix.
The cytochrome b gene has been proved as an efficient tool with high power of discrimination for species identification and characterization in both taxonomy and forensic science (Kuwayama & Ozawa, 2000; Saif *et al*., 2012), and is also used in studies of molecular evolution (Prusak *et al*., 2004). The gene length is 1140 bp and has some stable sequences which were used for suggestion of universal primers for typical PCR-based methods (Parson *et al*., 2000). The application of DNA data to phylogenetic re-construction has led to the collection of diverse molecular data sets to test taxonomic hypotheses (Rose, 2005 & Archibald).This practice evidently leads to well- supported, highly resolved trees, compared with individual partitions. How-ever, the acquisition of such data sets is an expensive and time-consuming process. If it is accepted that the best estimate of phylogeny is taken to be the simplest ex-plantation of all the relevant data (i.e., the simultaneous analysis) (Miller *et al*., 1997), the PB Sallows an evaluation of phylogenetic accuracy of all partitions. So, in the present study phylogenetic analysis of snake using cytochrome b gene sequence was investigated.

MATERIAL AND METHODS

Collection of Samples

Blood samples were also provided from the Mumbai Zoological Gardens, collected from individual live Sand boa snake and Rat snake encountered in and around residential areas. Ventral scale clip samples and shed skins were taken from captive held specimens of known geographic origin. Whole blood samples were collected aseptically in sterilized vacationer tubes containing EDTA as anticoagulant, stored at -20°C until DNA extraction.

RESULTS AND DISCUSSION

DNA extraction and quantification

DNA Extraction from 2-3 scale was carried out using Gen- Elute mammalian genomic DNA miniprep Kit (Sigma- Aldrich). Concentration of DNA was determin ed using UV-1800 spectrophotometer (Schimadzu Corporation). The DNA was stored at -20^{0}C for further use.

PCR amplification

PCR amplification was performed using Biometra thermal cycler.The PCR mixture contained 2.5µl of 10X buffer, 1µl of each primer (diluted 10 times), 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µlTemplate DNA. Primers used for cyt bregion amplification were Snk- F: 5' TGAGGACAAATATCATTCTGAG 3' and Snk-R: - 5' TAGGCGAATAGGAAGTATCA 3' (Dubey *et al*.,
2010). The PCR amplification cycle consisted of, a cycle of 5 min at 94 °C; 30 cycles of 45 sec at 94 °C, 45 sec at 45°C,1 min and 30 sec at 72 °C; and 1 cycle of 5 min at 72 °C.

Gel electrophoresis

Gel electrophoresis was performed using 1.0% agarose to analyze the size of amplified PCR product. The size obtained was approx. 431bp for cytochrome b region (Figure 4).

DNA sequencing

The PCR product was purified using AxyPrepPCR Clean up kit (AP-PCR-50).It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram obtained. The primer used was Snk-F:5' TGAGGACAAATATCATTCTGAG 3'as sequencing primer.

Bioinformatics analysis

The DNA sequences were analyzed using online nBLAST (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI).The BLAST results (Table 1 & 2) were used to find out evolutionary relationship of snake. Altogether eleven sequences, including sample were used to generate phylogenetic tree. The tree had been generated in MEGA (Figure 1 & 2).

Table 1. Phylogenetic analysis of Sand boa.

S. No	Description	Max score	Max ident	Accession
1.	*Eryxcolubrinusloveridgei* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	398	89%	U69812.1
2.	*Eryxconicus* cytochrome b (Cytb) gene, partial cds; mitochondrial	375	88%	GQ225658.1
3.	*Eryxcolubrinuscolubrinus* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	348	86%	U69811.1
4.	*Eryxconicus* cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	339	88%	U69817.1
5.	Eryxjaculus cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	309	87%	U69821.1
6.	Eryxconicus cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	307	88%	U69816.1
7.	Epicratesmonensis cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, complete cds	294	84%	U69792.1
8.	Epicratesmonensis cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, complete cds	294	84%	U69790.1
9.	Morelia amethistina cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	292	85%	U69847.1
10.	Python regius cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds	285	84%	U69856.1

Sample no. Sand boa *Gene Sequences*: > Sand boa Snake, Sequence length: 367.

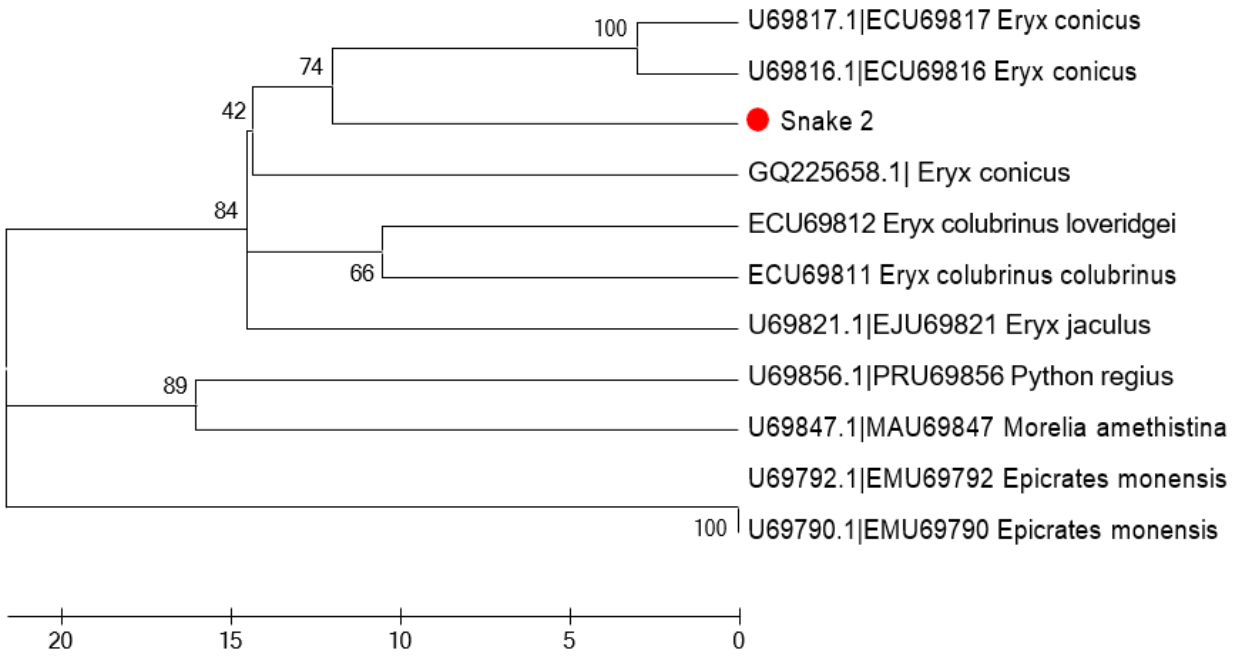

Figure 1. Phylogenetic tree for Sand boa.

Table 2. Phylogenetic analysis of Rat snake.

S.No	Description	Max score	Max identity	Accession no.
1.	*Ptyasmucosus* CAS 208434 cytochrome b (cytb) gene, complete cds; mitochondrial	392	89%	AF471054.1
2.	*Ptyasmucosus* cytochrome b (Cytb) gene, partial cds; mitochondrial	381	88%	GQ225655.1
3.	*Dinodonrufozonatum*LSUMZ 44977 cytochrome b (cytb) gene, complete cds; mitochondrial	243	83%	AF471063.1
4.	*Dinodonrufozonatum*isolate china cytochrome b (cytb) gene, partial cds; mitochondrial	220	82%	JF827672.1
5.	*Coluber constrictor* voucher KJI 3 cytochrome b gene, partial cds; mitochondrial	198	80%	EU180478.1
6.	*Hypsiglenajanitexana* voucher CAS 228960 mitochondrion, complete genome	193	80%	EU728592.1
7.	*Contiatenuis* voucher MVZ 232671 cytochrome b (cytb) gene, completecds; mitochondrial	189	80%	GU112395.1
8.	Contiatenuis voucher MVZ 208160 cytochrome b (cytb) gene, complete cds; mitochondrial	189	80%	GU112393.1
9.	Contiatenuis voucher MVZ 208158 cytochrome b (cytb) gene, complete cds; mitochondrial	189	80%	GU112392.1
10.	Contiatenuis voucher CAS 205652 cytochrome b (cytb) gene, complete cds; mitochondrial	189	80%	GU112385.1

Sample no. Rat snake *Gene Sequences*: >Snake Rat snake, Sequence length: 359.

AGGGGGAGGGGGGAGGAGGGGTGCTTCAATTTGGGGATACCAACTGCTAACCCGGTTGGTTGGG
CCTACGG
TTTATCCTTCCGGGAGTATCATGTCAATGTCCTCAATCCACATCATACTGCTTCACACAGAAGATCAAG
CAAC
CCGCTAGGAACAAATTCAGACATGGACAAAATTCCATTTCACCCATACCACTCCCACAAAGATAT
CCTAATA
CGAACGGTATTAATTACCATAATATTTACTATTATAGCATTCACCCCAAACATAGGTGATGAGCC
AGAAAAT
GTCTCAAAAGCTAATCCAATAGTCGCACCACAACACATTAAACCAGAAGGATACTTCCTAGTCGC
CTAAGA

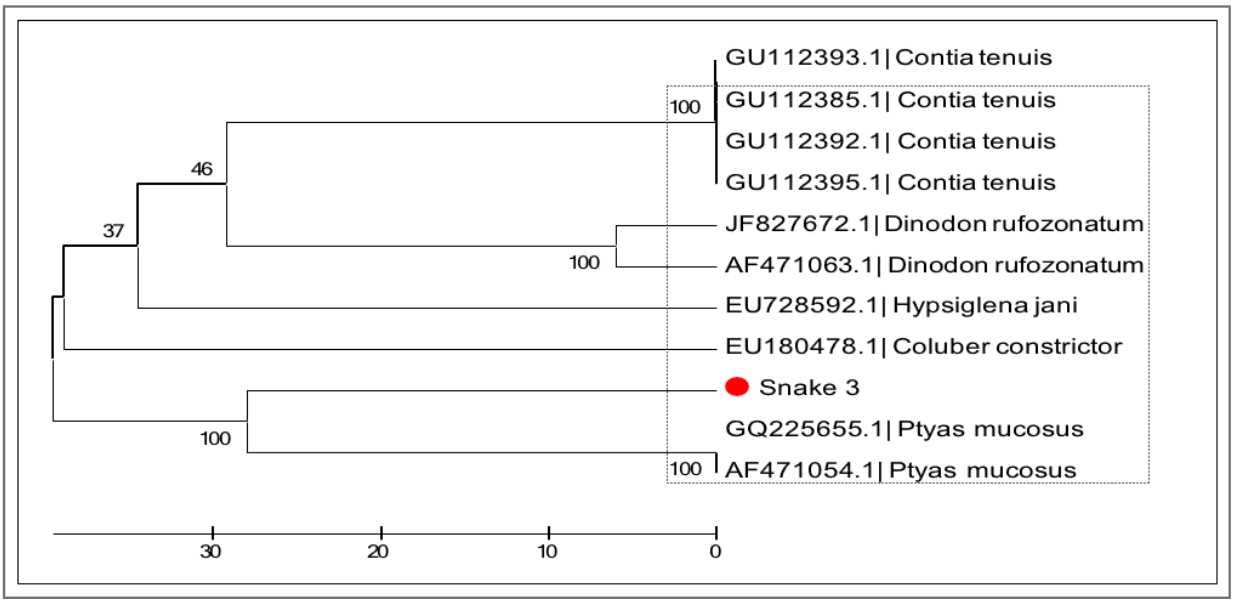

Figure 2. Phylogenetic tree for Rat snake.

Two recent molecular studies (Keogh *et al*., 1998) [partial16S and cytochrome b mtDNA sequences]; [amino acid sequences of the venom proteins PLA2 and NXS]) have explored the phylogenetic relationships within the snake family Elapidae, a major group of venomous snakes containing nearly 300 species in approximately 60 genera (Golay *et al*., 1993); herein we use Elapidae in the broad sense to include both terrestrial and marine species, whereas (Golay *et al*., 1993) place the marine species in a separate family Hydrophiidae). The two studies agree that the marine and Australo-Melanesian species are collectively monophyletic. Initial phylogenetic analyses using maximum likelihood joined acrodonts with snakes instead of with iguanids. The affinities of snakes and acrodonts were thus analyzed separately in subsequent analyses. Furthermore, the most fast-evolving taxa were removed by creating a distance matrix based on themtREV+I+8 models and compared distances between each squamate taxon and the turtles. Any squamate taxon that had a distance of 1.0 or more was removed. The taxon sampling may have been compromised but it was only compromised within, not across, lineages. In addition, fast- evolving taxa only mask the true phylogenetic signal of the data and, as was evident, increase the likelihood of LBA (Baurain *et al*., 2007). These results are congruent with previous phylogenetic studies with respect to the affinities of lizard lineages. These results support a recent mitogenomic study that also place snakes with the "Laterata" (Kumazawa, 2007). These results are in contrast to nuclear gene studies that phylogenetic analysis of snake like *Eryxcolubrinus loveridgei* and *Ptyas mucosus.*

CONCLUSION

The molecular phylogeny of sample had been determined by analyzing cytochrome b gene sequence. On the basis of position of sample sequence of given snake samples in the phylogenetic tree, the following results were observed.

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