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DNA barcoding of *Python molurus* using cytochrome b gene sequences analysis

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Abstract
The DNA sequence diversity of *Python molurus* was investigated using the mitochondrial DNA 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 *Python molurus.*

Keywords: *Python molurus,* DNA sequence, cytochrome b, molecular phylogeny

Introduction
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) [1]. 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) [3]. 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 (Pereira *et al.,* 2008) [8].
Molecular taxonomic approaches using DNA barcoding could aid in snake identification as well as studies of biodiversity. Here a non-invasive sampling method using DNA barcoding is tested using skin exuviates. Taxonomically authenticated samples were collected and tested for validation and comparisons to unknown snake exuviate samples. This approach was also used to construct the first comprehensive study targeting the snake species from Maharashtra state in India. Analysis of mitochondrial DNA (mtDNA) sequences is useful for phylogenetic studies. The mtDNA is independent, simpler than genomic DNA, and is of maternal inheritance and has no recombination in all vertebrates, so the sequence of mtDNA is more conservative (Rokas *et al*., 2003) [10]. The rate of base substitution on mtDNA is 5-10 times relative to nuclear gene which resulted in an accumulation of base substitutions over a long period of time, and enabled discrimination of a wide variety of birds, even closely related species belonging to the same families and genera (Russell *et al.,* 2000) [11].
Among mitochondrial genes, *cytochrome b* (mt *Cytb*) 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 and Ozawa, 2000; Saif *et al*., 2012) [5, 12], and is also used in studies of molecular evolution (Prusak *et al*., 2004) [9]. 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) [7]. Barcoding of Python was done by amplifying mitochondrial cytochrome b region. Two primers specific to cytochrome b region used in this study were Snk F and Snk R in order to amplify approx. 400bp sequence of Python cytochrome b gene.

Materials and Method
Blood samples: Blood samples were also provided from the Mumbai Zoological Gardens, collected from individual live pythons 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 vacutainer tubes containing EDTA as anticoagulant, stored at -20°C until DNA extraction.

DNA extraction and quantification: DNA Extraction was carried out using Genelute Mammalian Genomic DNA extraction kit (Sigma, G1N70-1KT). 25mg of tissue was minced and transferred to 1.5ml microcentrifuge tube. 180µl

of Lysis solution T and 20 µl of proteinase K were added. The samples were mixed and incubated at 55^0C to digest the tissue completely. 20 µl of RNase A solution was added and incubated at room temperature for 2min. Then 200µl of lysis solution C was added and incubated at 70^0C for 10 min. The column was prepared for binding by adding 500µl of Column preparation solution to each pre-assembled Gen Elute Miniprep Binding Column and centrifuge at 12,000 rpm for 1 min. 200µl of ethanol was added to the lysate and mixed by vortexing. The entire lysate was transferred into the treated binding column and centrifuge at 10,000rpm for 1 min. The binding column was then placed in fresh 2ml collection tube. 500µl of Wash solution was added to the binding column and centrifuge at 10,000 rpm for 3min. This step was repeated twice. The column was again transferred to a new tube. 200µl of elution buffer was added directly into the centre of the binding column and centrifuge at 10,000rpm for 1min. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu Corporation A11454806498). The DNA was stored at -20^0C for further use.

PCR amplification: The DNA isolated from Python was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler (T-Personal 48). The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer (Table 2), 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consist of, a cycle of 5 min at 94°C; 35 cycles of 1min at 94°C, 1 min at 48°C, 2 min at 72°C; and additionally 1 cycle of 7 min at 72°C. The reagents used are procured from GeNei (Table 3).

Gel electrophoresis: Gel electrophoresis was performed using 1.0% agarose (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 400bp for cytochrome b region (Figure 2).

DNA sequencing: The PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50). 100µl of PCR-A buffer was added to the 25µl of reaction. The sample was mixed and transferred to column placed in 2ml collection tube and centrifuge at 10,000 rpm for 1min. the filtrate was discarded.700µl of W2 buffer was added to the column and centrifuge at 10,000rpm for 2min. This step was repeated twice. The column was transferred to a new tube. 25µl of Eluent was added into the column and incubated at room temperature for 2min. Then centrifuge at 10,000rpm for 5min. It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained. For sequencing of PCR product Snk F - 5' TGAGGACAAATATCATTCTGAG 3' sequencing primer was used.

Bioinformatics analysis: The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of Python. Altogether Twenty sequences, including sample were used to generate phylogenetic tree (figure 1). The tree was constructed by using MEGA 5 software (Saitou N. and Nei M., 1987; Felsenstein J. 1985) [13, 4].

Results and Discussion

Sample no.: Python

Partial cytochrome b gene Sequence (372bp)

>Python

GCCGTACCATACTTAGGCACAACCCTAACAACCTGG
TTATGAGGAGGATTCGCAATCAATGACCCCACCCTC
ACACGATTCTTTGCACTACATTTCATCCTACCATTCG
CAATCATCTCCATATCATCACTACACATTATCCTACT
CCACGAAGAGGGATCTAGCAACCCACTAGGGACAAA
CCCAGACATCGACAAAATCCCATTCCACCCTTACCAC
TCATACAAAGACCTACTCTTCCTGACCCTAATAATCC
TATTTATACTCATCATCGTCTCATTCTTCCCTGATATC
TTCAACGACCCAGACAACTTCTCAAAAGCCAATCCA
CTAGTTACACCCCAACACATTAAACCAGAGTGATAC
TTCCAA

Sequence length: 372bp

Table 1: Phylogenetic neighbors of Python based on cytochrome b gene sequence

Description	Max score	Query cover	Ident	Accession
Python molurus molurus cytochrome b (cyt b) gene, partial cds; mitochondrial	654	99%	99%	FJ936560.1
Python molurus molurus cytochrome b (Cytb) gene, partial cds; mitochondrial	645	99%	98%	GQ225654.1
Python molurus cytochrome b (cytb) gene, mitochondrial gene encoding mitochondrial protein, partial cds >gb|AY099983.1| Python molurus cytochrome b (cytb) gene, partial cds; mitochondrial	645	99%	98%	U69853.1
Python molurus molurus mitochondrion, complete genome	601	98%	96%	HM581978.1
Python bivittatus isolate PM031 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401163.1
Python bivittatus isolate PM030 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401162.1
Python bivittatus isolate PM029 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401161.1
Python bivittatus isolate PM028 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401160.1
Python bivittatus isolate PM027 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401159.1
Python bivittatus isolate PM026 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401158.1
Python bivittatus isolate PM025 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401157.1
Python bivittatus isolate PM024 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401156.1
Python bivittatus isolate PM023 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401155.1
Python bivittatus isolate PM022 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401154.1
Python bivittatus isolate PM021 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401153.1

Python bivittatus isolate PM020 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401152.1
Python bivittatus isolate PM018 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401150.1
Python bivittatus isolate PM017 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401149.1
Python bivittatus isolate PM016 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401148.1
Python bivittatus isolate PM015 cytochrome b (cytb) gene, partial cds; mitochondrial	584	99%	95%	JX401147.1

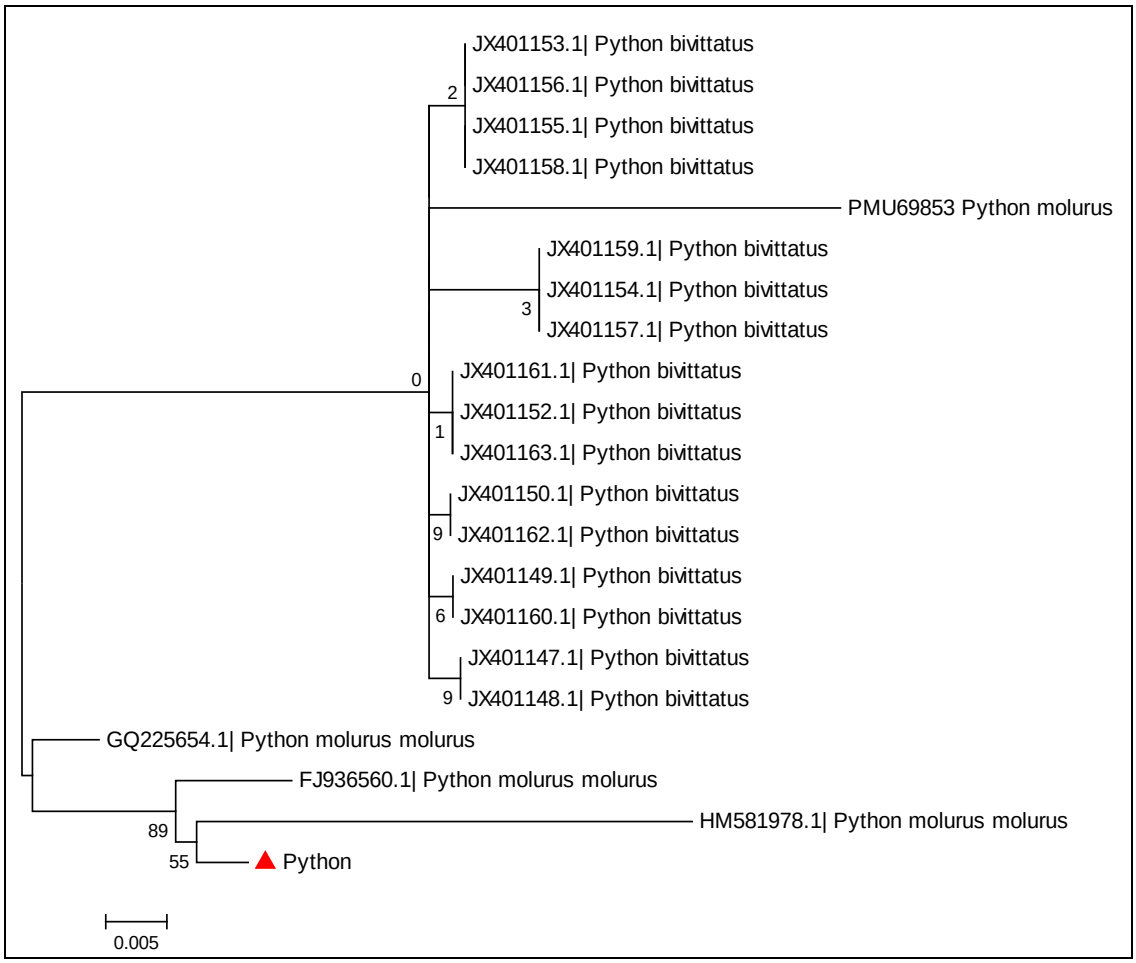

Fig 1: Phylogenetic tree for Python using cytochrome b gene sequence

Table 2: Primers used for cytochrome b gene amplification

Primers	Primer Sequence (5'-3')
Snk F (Forward)	TGAGGACAAATATCATTCTGAG
Snk R (Reverse)	TAGGCGAATAGGAAGTATCA

* Bhawna Dubey *et al.* 2010 [2]

Conclusion

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 *Python molurus*

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