# DNA bar coding of meat and bone samples of suspected endangered species: A case study

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# **Abstract**

DNA bar coding is a powerful tool for species identification involving sequence analysis of mitochondrial DNA. We describe here a case where an animal was killed and eaten. Cooked meat and bone pieces which could not be identified on morphological and biochemical grounds were sent for identification of species. Commonly used heating methods for cooking meat e.g. boiling can significantly affect the quality and yield of DNA extracted from meat, making it difficult to apply DNA based techniques for species identification. However, in the present case, cooked meat and bones were analyzed successfully on the basis of cytochrome oxidase subunit I (coi) marker. The query sequence obtained was compared to that of the reference in NCBI Gene bank data base and BOLD database. Sequence analysis revealed that the samples were of Bengal monitor lizard (Varanus bengalensis) included in schedule I part II of Wildlife Protection Act 1972, Government of India.

Key Words: Mitochondrial DNA, PCR amplification, cytochrome oxidase I, endangered species.

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# **INTRODUCTION**

The Bengal Monitor lizard or Indian Monitor lizard (Varanus bengalensis) is treated as an endangered species and is accorded protection under the Wildlife Protection Act, 1972 of Government of India. Because of its endangered status, it is also enlisted in Appendix I of CITES (Convention for International Trade in Endangered Species of Flora and Fauna). These aquatic animals play the role of scavengers in keeping the water bodies free from harmful insects and bugs. Once found abundant along the water bodies, their number is significantly decreasing due to poaching. Poaching of

monitor lizard is done for their skin and flesh. Skin is used in musical instruments while flesh is used as food. However, it is a cognizable offence to sell such products and the offender may get up to 7 years of imprisonment. If the conviction rate of accused involved in poaching is increased and there is a control on illegal poaching, then conservation of species would be a success. Definite identification of species is necessary for conviction under various wild life protection acts. Generally in cases of suspected poaching, the only available evidence is pieces of meat, skin or bones. In such cases, species identification can be done using molecular techniques. The application of DNA based technologies to the investigation of wildlife crime enables analysis of trace evidence samples. Mitochondrial DNA (mtDNA) testing has become a standard procedure in species identification as there is no recombination of mtDNA. All maternal descendants will have the same mitochondrial DNA sequence with the exception of mutations and all loci will be linked<sup>1, 2</sup>. Additionally, there are multiple copies of mitochondrial DNA per cell compared to only two copies of nuclear DNA<sup>3</sup>. For forensic species identification, genetic loci on mitochondrial DNA are derived from taxonomic and phylogenetic studies<sup>4</sup>. In the present case,

How to site this article: V J Thakare, P S Kene, P V Thakare, A A Pande. DNA bar coding of meat and bone samples of suspected endangered species: A case study. *MedPulse International Journal of Forensic Medicine*. February 2017; 1(2): 18-21. https://www.medpulse.in/Forensic%20Medicine/ (accessed 18 February 2017).  $\begin{array}{c} \mbox{cytochrome oxidase I}^{5,\,6} \mbox{ was used as marker. It is adopted} \\ \mbox{by} & \mbox{Barcode} & \mbox{for} & \mbox{Life} & \mbox{Consortium} \\ \mbox{http://www.boldsystems.org}^{\,7,\,8}. \end{array}$ 

# Brief history of case

Chief Conservator of Forest called Deputy Director of Regional Forensic Science Laboratory, Amaravati, stating that there was a case of poaching of wild animal suspected to be of Indian Monitor Lizard. 4 -5 Monitor Lizards were captured, killed, eaten and the left over material was thrown away. Collection of samples was a major problem for the Range Forest Officer and hence forensic experts were called in the midnight. The team reached the spot and found a gunny bag in which the animals were kept, wooden piece on which the animals were slaughtered, leftover cooked meat, uncooked meat with fatty tissue and leftover bones from cooked meat. Blood and small tissue pieces approximately 200 - 300 mg were collected from wooden piece and tissue matter from fatty tissue was separated and collected. Spinal cord and bone piece from which there was a possibility of recovery of DNA were carefully taken from approximately 30 -40 bone pieces. All the sample collection was carried out by the forensic team from midnight till dawn. Collected samples were sent to Regional Forensic Science Laboratory, Amravati for species identification.

# **Extraction of DNA from samples**

DNA from meat sample, bone, spinal cord, blood detected on wooden piece and gunny bag and cooked meat sample was extracted using manual extraction protocol. DNA was successfully isolated from meat sample, cooked meat sample, bone sample and spinal cord. No DNA was isolated from blood detected on wooden piece and gunny bag. Agarose gel electrophoresis was used for separation of genomic DNA and the DNA bands were visualized on gel documentation system. (Alpha Innotech, USA).

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Figure 1: Lane 1: water blank, Lane 2: wooden piece, Lane 3: meat sample, Lane 4: cooked meat, Lane 5: bone piece, Lane 6: spinal cord

# DNA amplification using Polymerase Chain Reaction (PCR)

Cytochrome oxidase I gene (coi) of mitochondrial region was used for PCR amplification using universal primer pair that consistently amplified around 700 bp fragment of *coi* across the broadest array of animal orders. Primer pairs used for DNA amplification targeted single copy mitochondrial DNA. Thermal cycling was performed in 0.2 ml thin walled PCR tubes with 20  $\mu$ l reaction volume. PCR products were analyzed by electrophoresis on 1.2% agarose gel and visualized using ethidium bromide.



**Figure 2:** DNA amplification of *coi* locus of mitochondria Lane 1: 100 bp ladder, Lane 2: wooden piece, Lane 3: meat sample, Lane 4: cooked meat, Lane 5: bone piece, Lane 6: spinal cord

# **DNA** sequencing

Amplified DNA i.e. *coi* gene obtained was sequenced in ABI 3500 Genetic Analyzer using ABI Big Dye TM Terminator Cycle sequencing kit by Chromous Biotech Pvt. Ltd., Bengaluru, Karnataka, India.

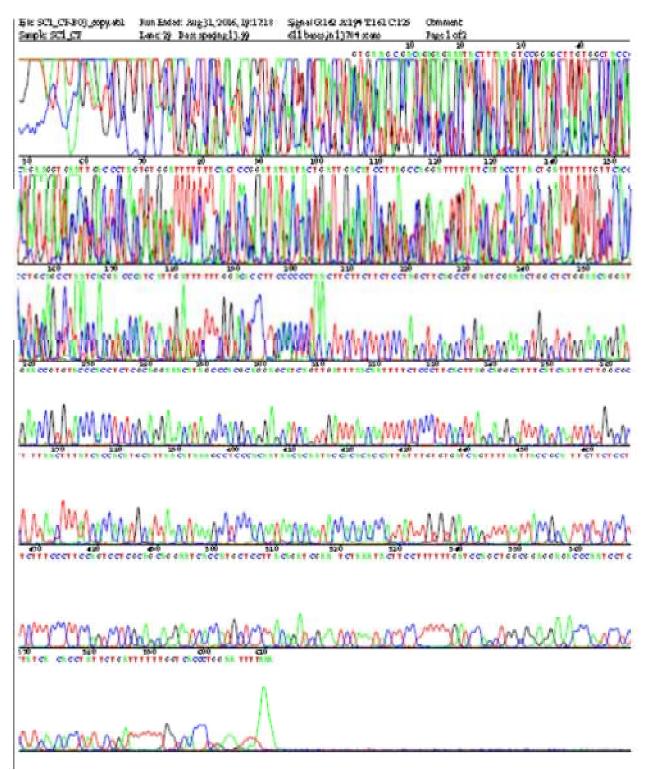


Figure 3: Nucleotide sequence chromatogram of meat sample

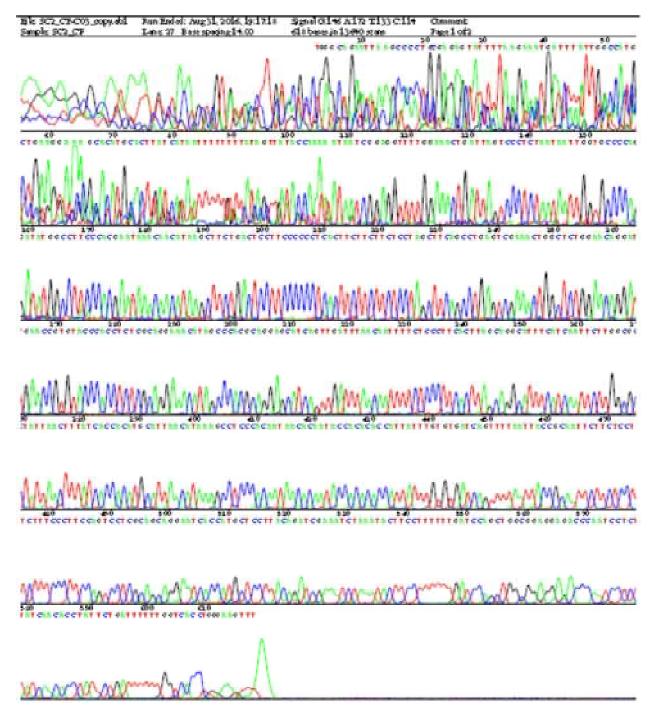


Figure 4: Nucleotide sequence chromatogram of bone piece

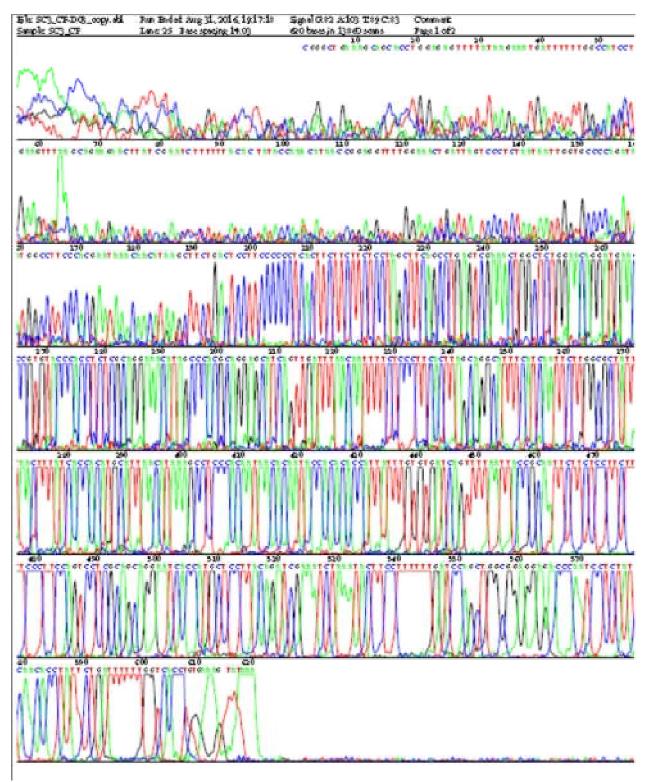


Figure 5: Nucleotide sequence chromatogram of cooked meat sample

All the sequences obtained were same as compared in Clastal W. From analysis, it was concluded that nucleotide sequence of all the samples was same and belonged to same species of animal. The sequences obtained from all samples were searched using BLAST and the sequences showed match of 99-97% with *Varanus bengalensis* i.e. Monitor Lizard.

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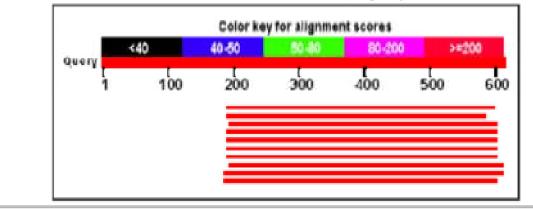
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# ■ Graphic Summary





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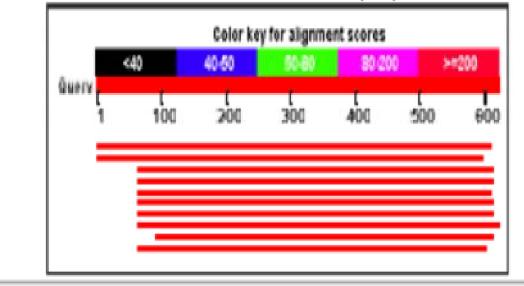


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BLAST search of bone piece

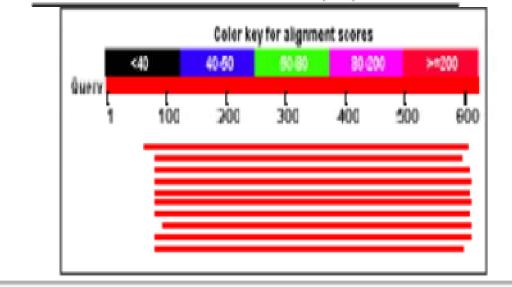
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# Graphic Summary

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### Figure 9:

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# BLAST search of bone sample.

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Forward and Reverse sequence were combined to form a Contig sequence in CAP –Contig Assembly Program of Bioedit software (v. 7.0.9.0)<sup>9</sup> forming Contig query sequence of 700 bp of *coi* sequence. Following figure shows the alignment in Bioedit of samples and sequences from NCBI and BOLD<sup>10, 11</sup>.

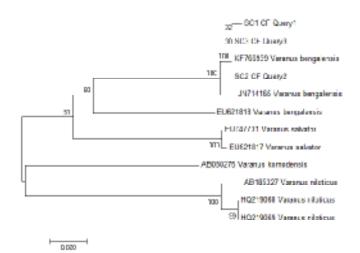
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KF766939_Varanus_bengalensis	
JN714165_Varanus_bengalensis	
SC3_CF_Query3	aa
EU621818_Varanus_bengalensis	
EU747731_Varanus_salvator	
EU621817_Varanus_salvator	
AB185327_Varanus_niloticus	
HQ219068_Varanus_niloticus	
HQ219069 Varanus niloticus	
AB080275 Varanus komodensis	

Result of Contig sequence of Varanus bengalensis in CAP-Contig Assembly Program of Bioedit Software.

## Dendrogram Tree

BLAST computes a pair wise alignment between a query and the database subject sequences searched. For sequence tree presentation, an implicit alignment between the database sequences was constructed, based upon the alignment of those database subject sequences to the contig query. Figure shows the dendrogram tree for matching of the unknown sample's nucleotide sequences with known sequences.



The meat, bone and cooked meat sample were analyzed for *coi* nucleotide sequences. It was found that the nucleotide sequences for *coi* were same and belonged to same animal. Upon detailed analysis of the sample, it was found to be of *Varanus bengalensis* (also called as monitor lizard) based on DNA barcode analysis as well as the nucleotide sequence analyzed from NCBI (National Centre for Biotechnology Information, USA) and BOLD (Barcode of Life Database). Phylogenic study shows the similarity on comparison with that of the reference data. From the dendrogram, it is clear that the species came in one clad of *Varanus bengalensis*. The contig match with *Varanus bengalensis* species demonstrated that the sample was of *Varanus bengalensis*.

## DISCUSSION AND CONCLUSION

Varanidae is a family of lizards that contains the living genus *Varanus* which includes monitor lizards. Monitor lizards are generally large reptiles with long necks, powerful tails and claws and well developed limbs. In India, four kinds of monitor lizards occur. They are:

- *V. griesus* or Desert monitor lizard distributed mainly in Indus valley and is largely restricted to sandy desert, being uncommon in clay deserts. It is also found in central and western Pakistan.
- *V. flavescens* or Yellow monitor lizard found mainly in flood plains of Indus, Ganges and Brahmaputra rivers of Pakistan, North and Northeast India, Nepal and Bangladesh.
- *V. salvator* or Water monitor lizard whose distribution extends from Northwest India, Sri Lanka across Burma to Combodia in the west and up to the Philippines in the east.
- *V. bengalensis* or Bengal monitor lizard mainly found throughout India, Sri Lanka, Bangladesh, Afghanistan and Pakistan<sup>12, 13</sup>.

All the Indian species are classified under CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) appendix I and Wildlife Protection Act (1972) schedule I, which outlaws international commercial trade of these species and also catching or killing of such species in India amounts to imprisonment up to seven years and fine not less than ten thousand rupees<sup>14, 15</sup>. In this case, identification was done with the help of COI gene. This method is reliable as it showed versatility in its ability to use a single conserved primer pair to accurately identify, if conspecific sequences were available in database.

Careful and in-time collection of samples by the forensic team helped in precise analysis of the samples which otherwise was not possible by the forest officials.

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