

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

V J Thakare^{1*}, P S Kene¹, P V Thakare², A A Pande¹

¹Deputy Director, Regional Forensic Science Laboratory, Home Department, State of Maharashtra, Amravati-444601, Maharashtra, INDIA.

²Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati, Maharashtra, INDIA.

Email: dydir.rfslamravati@maharashtra.gov.in

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.

*Address for Correspondence:

Dr. Vijay J. Thakare, Deputy Director, Regional Forensic Science Laboratory, State of Maharashtra, Home Department, Amravati-44460, Maharashtra, INDIA.

Email: dydir.rfslamravati@maharashtra.gov.in

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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^{1, 2}. Additionally, there are multiple copies of mitochondrial DNA per cell compared to only two copies of nuclear DNA³. For forensic species identification, genetic loci on mitochondrial DNA are derived from taxonomic and phylogenetic studies⁴. In the present case,

cytochrome oxidase I^{5,6} was used as marker. It is adopted by Barcode for Life Consortium <http://www.boldsystems.org>^{7,8}.

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).

1 2 3 4 5 6

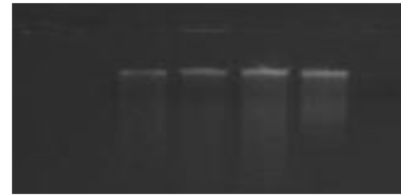


Figure 1: Lane 1: water blank, Lane 2: wooden piece, Lane 3: meat sample, Lane 4: cooked meat, Lane 5: bone piece, Lane 6: spinal cord

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 µl reaction volume. PCR products were analyzed by electrophoresis on 1.2% agarose gel and visualized using ethidium bromide.

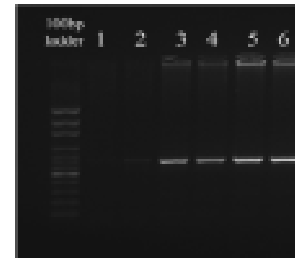


Figure 2: DNA amplification of *coi* locus of mitochondria Lane 1: 100 bp ladder, Lane 2: wooden piece, Lane 3: meat sample, Lane 4: cooked meat, Lane 5: bone piece, Lane 6: spinal cord

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.

[BLAST](#) » [blastn suite](#) » RID: XZRUVW2X01R

BLAST Results

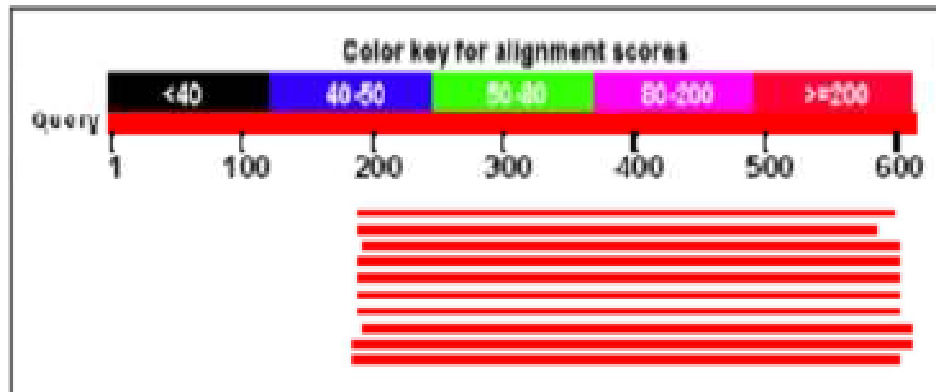
SC1-CF-B03-copy

RID [XZRUVW2X01R](#) (Expires on 09-20 20:51 pm)

Query ID	ic Query_100705	Database Name	nt
Description	None	Description	Nucleotide collection (nt)
Molecule type	nucleic acid	Program	BLASTN 2.5.0+
Query Length	611		

[Graphic Summary](#)

Distribution of 10 Blast Hits on the Query Sequence



Descriptions

Sequences producing significant alignments.

Descriptions	Max Score	Total Score	Query cover	E value	Ident	Accession
<i>Varanus bengalensis</i> vouchae 5-L9234 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	100	100	100%	0.0	99%	MF764081.1
<i>Varanus bengalensis</i> orstedii V9-JR021-05-1 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	85.0	85.0	94%	0.0	97%	JF752388.1
<i>Varanus bengalensis</i> orstedii V001 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	47.9	47.9	100%	4e-131	97%	KJ830238.1
<i>Varanus zoolae</i> mitochondrial DNA, complete genome	44.1	44.1	87%	1e-119	95%	AB682801.1
<i>Varanus zoolae</i> haplotype mitochondrial DNA, complete genome	43.7	43.7	87%	1e-119	95%	AB682802.1
<i>Varanus zoolae</i> mitochondrial, complete genome	43.7	43.7	87%	1e-119	95%	AB682803.1
<i>Varanus zoolae</i> orstedii V301 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	43.4	43.4	100%	2e-117	95%	KJ830239.1
<i>Varanus mackinnoni</i> mitochondrial DNA, nearly complete genome, region 12	39.0	39.0	87%	1e-109	92%	AB682804.1
<i>Lampaka sepioides</i> mitochondrial, complete genome	37.9	37.9	100%	1e-109	90%	FJ754581.1
<i>Melanoscolobrodia</i> COI M1 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial	37.0	37.0	87%	2e-99	90%	MF764082.1

Alignments

Varanus bengalensis vouchae 5-L9234 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial
 Sequence ID: MF764081.1 Length: 825 Number of Matches: 1
 Range: 1-275 to 825

Score	Expect	Matches	Gaps	Strand	Frame
100 bits (170)	0.0	403/411 (98%)	4/411 (1%)	Plus/Plus	

Features:

Query	1-9	G A C A C C T T C C C C C C T A A C T C T T C T C T C C T A G C T T C A C C C T G A C T C G A A C T C C C T C T G	2-9
Subject	275	G A C T C C T T C C C C C C T C A C T	334
Query	2-9	T T	340
Subject	331	T T	394
Query	30-9	T T	357
Subject	395	T T	414
Query	36-9	A G C T	477
Subject	411	A C T	511
Query	7-9	T A T	485
Subject	111	T T	524
Query	4-7	C A G C A G G A T T G A C C A T G C T C C T T A C A G A T G G A A T C T A A T A C T T C T T T T T T T T T T T T T T T T T T T	545
Subject	171	C C C A G G A T T G A C C A T G C C T T A C A G A T G G A A T C T A A T A C T T C T T T T T T T T T T T T T T T T T T T	613
Query	5-6	C T C	595
Subject	631	C T C	691

Varanus bengalensis orstedii V9-JR021-05-1 cytochrome c oxidase subunit I (COI) gene, partial cds, mitochondrial
 Sequence ID: JF752388.1 Length: 825 Number of Matches: 1

Figure 7: BLAST search of meat sample

[BLAST](#) > [Nucleotide](#) > [RD-XLLAXKKD14](#)

BLAST Results

BC2-CF-C08-nop;

RD: [XXXXXX5014](#) (Accession: [M21011.1](#))

Query ID: [U000000000](#)
Description: [Hb *c](#)
Molecule type: [mRNA](#)
Query length: 618

Database Name: [Hs](#)
Description: [Homo sapiens \(Hs\)](#)
Program: [BLASTN](#)

Graphic Summary

Distribution of 10 Best Hits on the Query Sequence

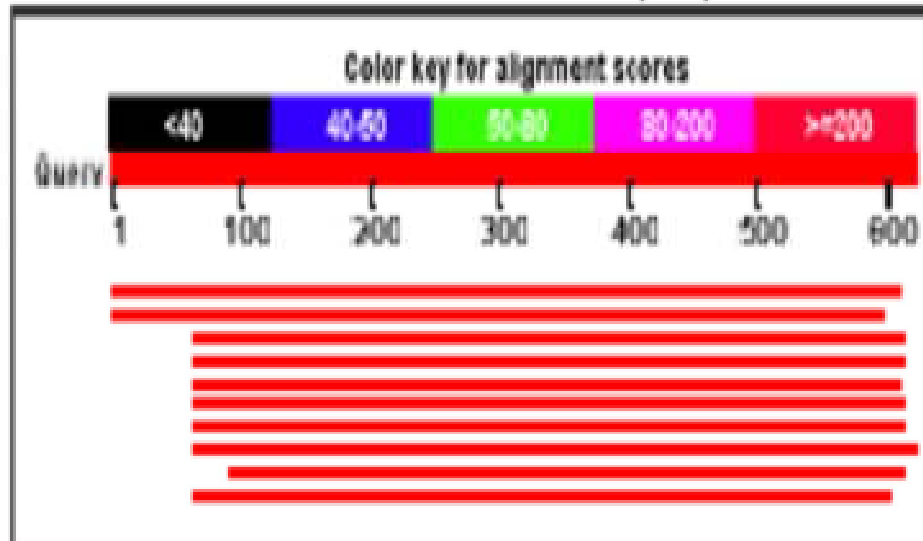


Figure 8:

Descriptions

Sequences producing significant alignments.

Description	Max Score	Total Score	Query Cover	E-value	Ident	Accession
<i>Vaccinium bengalense</i> voucher 5-L3224 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	284	284	97%	0.0	97%	AF128284.1
<i>Vaccinium bengalense</i> isolate V6-43 D1-CO-I cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	275	275	95%	0.0	94%	JF743282.1
<i>Vaccinium bengalense</i> isolate V601 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	243	243	97%	0.0	97%	JX422272.1
<i>Vaccinium</i> sp. mitochondrial DNA, complete genome	201	201	99%	2e-188	99%	AF091540.1
<i>Vaccinium</i> sp. human mitochondrial DNA, complete genome	200	200	97%	8e-187	99%	AF091538.1
<i>Vaccinium</i> sp. mitochondrial, complete genome	200	200	99%	2e-188	99%	AF091542.1
<i>Vaccinium</i> sp. isolate V501 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	204	204	97%	7e-188	98%	JX422273.1
<i>Vaccinium</i> sp. isolate mitochondrial DNA, nearly complete genome, region 12	244	244	99%	1e-190	99%	AF091543.1
<i>Miconia</i> sp. isolate COI cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	222	222	99%	4e-144	99%	MF301761.1
<i>Vaccinium</i> sp. isolate cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	218	218	99%	5e-142	99%	JX422270.1

Alignments

Vaccinium bengalense voucher 5-L3224 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial
 Sequence ID: AF128284 Length: 820 Number of Matches: 1
 Range: 1, 22 to 820

Score	Expect	Identifiers	Query	Subject	Frames
284 bits (1000)	0.0 (0)	5.24E04 (97%)	2804 (97%)	PlusPlus	

Features:

```

Query 1  CCAGCAATGACCCCTGACAGACTATTTAGGATGTTTCTTGGCCATGCTGAAAGCA 67
Sbjct 82  CCAGCAATTTAGCCGACCCGGGACTATTTAGGATATGATGCAATTTACAATGTGTGATGA 141
Query 61  TTTGGCTGCTGCTTTGTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTAT 121
Sbjct 147  TCCGGCTGCTGCTTTGTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTAT 201
Query 177  GGAATACGATTAATGCTCCGATTAATTTGGTGGCCGATTTGGCTTCCCGAATTAATC 181
Sbjct 267  GGAATACGATTAATGCTCCGATTAATTTGGTGGCCGATTTGGCTTCCCGAATTAATC 261
Query 187  AATGATAGCTCTGACTGCTTCCGCTGACTTCTTCTCTCTAGCTTCAAGCTGATGTC 241
Sbjct 267  AATGATAGCTCTGACTGCTTCCGCTGACTTCTTCTCTCTAGCTTCAAGCTGATGTC 321
Query 247  GAAATGCTGCTCTGAAACAGGATGAAAGCTGACCGAAGCTCTCCAGAGGAAACATAGCC 181
Sbjct 327  GAAATGCTGCTCTGAAACAGGATGAAAGCTGACCGAAGCTCTCCAGAGGAAACATAGCC 381
Query 307  GCAAGGCTGCTGATTTGATTAATGATTTTATTTTATTTTATTTTATTTTATTTTAT 161
Sbjct 387  GCAAGGCTGCTGATTTGATTTAATGATTTTATTTTATTTTATTTTATTTTATTTTAT 241
Query 367  CTGAGGCTGATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTAT 221
Sbjct 447  CTGAGGCTGATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTAT 301
Query 427  TTTCCAGCTGCTGATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTT 281
Sbjct 507  TTTCCAGCTGCTGATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTTTATTT 361
Query 487  CTTCGATGCTCTCCAGAGATGATGATGATGCTTCCAGAGATGAAATGCTAAATGCTTCC 341
Sbjct 587  CTTCGATGCTCTCCAGAGATGATGATGATGCTTCCAGAGATGAAATGCTAAATGCTTCC 641
    
```

BLAST search of bone piece

[BLAST](#) > [Nucleotide](#) > [NCBI](#)

BLAST Results

BC8-CF-008-009

RID: [Y000457016](#) (Accession: 09-20-2017 AM)

Query ID: [t10419_17673](#)
Description: No-c
Molecule type: nucleic acid
Query length: 620

Database Name: *
Description: [Nucleotide collection \(nr\)](#)
Program: [BLASTN 2.2.31](#)

Graphic Summary

Distribution of 10 Best Hits on the Query Sequence

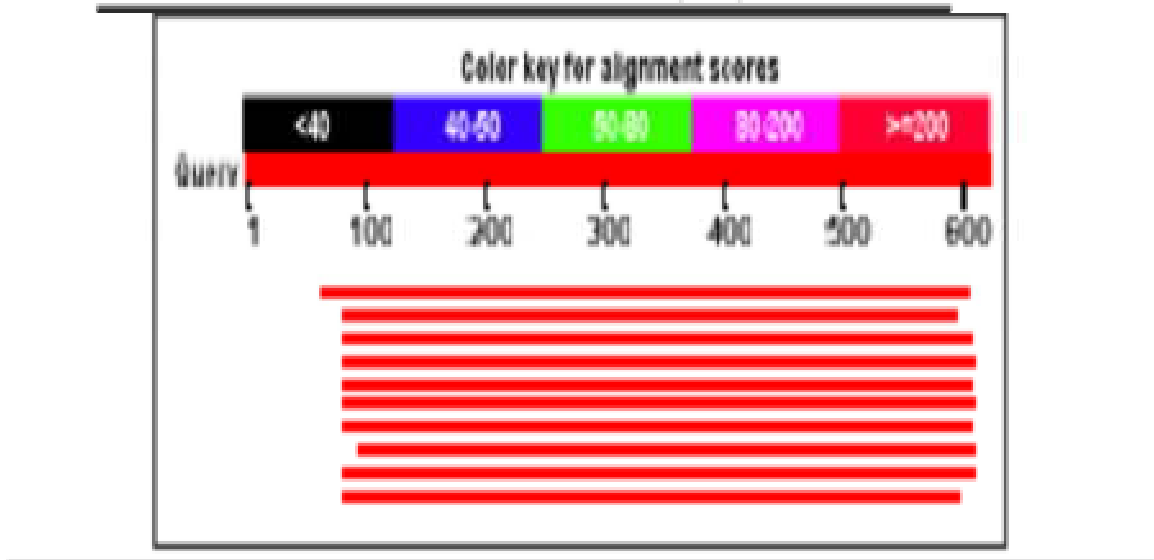


Figure 9:

Descriptions

Sequences producing significant alignments.

Description	Max Score	Total Score	Query Cover	E Value	Ident	Accession
Vacua bengalensis voucher SLS234 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	315	315	38%	0.0	98%	FJ218821
Vacua bengalensis voucher V6-4501-05-1 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	302	302	32%	0.0	98%	JF712345
Vacua bengalensis voucher V601 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	325	325	34%	4e-175	97%	KJ123456
Vacua sakhalina mitochondrial DNA, complete genome	598	598	34%	2e-188	35%	AB012345
Vacua sakhalina human mitochondrial DNA, complete genome	592	592	34%	3e-185	35%	AB012345
Vacua sakhalina mitochondrial, complete genome	590	590	34%	3e-185	35%	KJ123456
Vacua sakhalina isolate V501 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	503	503	34%	3e-184	35%	KJ123456
Vacua medusa mitochondrial DNA, nearly complete genome, region 102	528	528	32%	3e-145	35%	AB012345
Neoraneis quadrata COI III cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	315	315	34%	8e-142	32%	FJ218821
Vacua medusa cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial	313	313	33%	2e-141	32%	FJ218821

Alignments

Vacua bengalensis voucher SLS234 cytochrome oxidase subunit I (COI) gene, partial cds, mitochondrial
 Sequence ID: KJ123456 Length: 600 Number of Matches: 1
 Range: 145 to 325

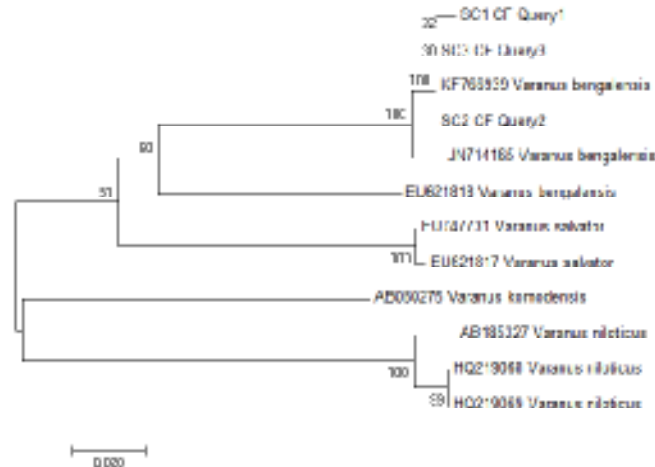
Score	Expect	Identical	Gaps	Strand	Frame
315 bits (1014)	0.0()	528/541 (97%)	35/41 (0%)	Plus/Plus	

Features:

```

Query 65  GCAGAGGACCTTATCGAATCTTTTTCACCTTATAACATTAACGGGAGTTTGGT 122
Sbjct 145  GCACATGCCTTATCATTTTTCATTTTATAGTTTAAOCATTTTATTCGGAGTTTGGT 204
Query 123  ATCTGATAGTCCCTTATTTTTCCTCCGAGATTTCCCTCCGACGATTAACATC 182
Sbjct 205  ATCTGATAGTCCCTTATTTTTCCTCCGAGATTTCCCTCCGACGATTAACATC 264
Query 183  ATTAAGCTTCTGACTCTCTCCCTCACTTCTTCTCTCTCTCTCTCTCTCTCTCT 242
Sbjct 265  ATTAAGCTTCTGACTCTCTCTCCCTCACTTCTTCTCTCTCTCTCTCTCTCTCT 324
Query 243  ACTGCTCTGCAACAGGATGACCGTGTACCACTCTCCGAGAAACAATACCCACA 302
Sbjct 325  ACTGCTCTGCAACAGGATGACCGTGTACCACTCTCCGAGAAACAATACCCACA 384
Query 303  GAGGATTCAGTTGATTTAAATTTTCTCCTTCACTTAGCAAGGATTCATCAATCT 362
Sbjct 385  GAGGATTCAGTTGATTTAAATTTTCTCCTTCACTTAGCAAGGATTCATCAATCT 444
Query 363  GGGCTATTAATTTTATGACCTATGCTTATGCTTATGCTTATGCTTATGCTTAT 422
Sbjct 465  GGGCTATTAATTTTATGACCTATGCTTATGCTTATGCTTATGCTTATGCTTAT 524
Query 423  CACGACCTATTTTCTGATGCTTTTATTTTCCGATTTCTTCTCTCTCTCTCTCT 482
Sbjct 525  CACGACCTATTTTCTGATGCTTTTATTTTCCGATTTCTTCTCTCTCTCTCTCT 584
Query 483  CCAGTCTCCGACACAGGAAACGATGCTCTCTTACGATCGAATCTAAATACCTT 542
Sbjct 625  CCAGTCTCCGACACAGGAAACGATGCTCTCTTACGATCGAATCTAAATACCTT 684
Query 543  TTGTATCCAGCTGGCCGAGGAGGACCAATCCCTCTATGACACCTATCTCTGATTT 602
Sbjct 685  TTGTATCCAGCTGGCCGAGGAGGACCAATCCCTCTATGACACCTATCTCTGATTT 664
    
```

BLAST search of bone sample.
 Same sequences were searched on the BOLD (Barcode of Life Database) for similarity index.



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). Phylogenetic 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 Cambodia 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^{12, 13}.

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^{14, 15}. 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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