Original Article

Molecular Forensics of Indian Wildlife: Species Identification through COI Gene Barcoding and Bioinformatics Analysis

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How to cite this article: Khushi Jain, Rakesh Mia. Molecular Forensics of Indian Wildlife: Species Identification through COI Gene Barcoding and Bioinformatics Analysis. Medico-Legal Update / Vol 25 No. 4, October - December 2025

Abstract

In wildlife forensics, precise species identification is essential, particularly in cases involving poaching, illegal wildlife trade, and biodiversity conservation. Molecular technologies are necessary for accurate forensic analysis since traditional morphological methods frequently fail when biological specimens are processed, incomplete, or degraded. Mitochondrial DNA (mtDNA) has proven particularly useful in these situations because of its large copy number, maternal mode of inheritance, and increased stability in damaged tissues. The cytochrome c oxidase subunit I (COI) gene is one of the most commonly used mtDNA markers for DNA barcoding due to its high interspecies variability, evolutionary conservation, and inclusion in extensive international databases.

This study examined the COI gene sequences of ten often encountered domestic and wild Indian species: *Elephas maximus* (elephant), *Bos gaurus* (gaur), *Bos taurus* (domestic cow), *Axis axis* (chital), *Axis porcinus* (hog deer), *Macaca mulatta* (rhesus macaque), *Pavo cristatus* (Indian peacock), *Sus scrofa* (wild boar), and *Panthera tigris* (tiger) and *Panthera pardus* (leopard). COI sequences that are publicly accessible were obtained from NCBI GenBank database. BLAST was used for sequence validation, and Clustal Omega was used for multiple sequence alignment. Using MEGA12, a neighbor-joining tree was constructed and pairwise distance analysis was performed to evaluate genetic distance and phylogenetic relationships.

Significant genetic divergence was found among the species under study, and the phylogenetic tree showed grouping patterns that aligned with established taxonomic relationships. These results demonstrate that COI-based DNA barcoding is a reliable method for differentiating between closely related species.

Keywords: species identification, cytochrome c oxidase subunit I (COI gene), mitochondrial DNA (mtDNA), DNA barcoding, wildlife forensics, bioinformatics tools

Introduction

Identification of species is essential to ecological monitoring, forensic science, and biodiversity protection.[1] When it comes to wildlife forensics, precise species identification is essential for preventing illicit poaching; stopping wildlife trafficking, and upholding laws protecting wildlife.[2]It is necessary for both legal processes and the preservation of endangered animals. The significance of accurately identifying species cannot be emphasized in a biodiverse nation like India, which is home to numerous endangered species.

Species identification in wildlife forensics has always depended on the morphological and anatomical analysis of physical remains. Bones, feathers or hair, teeth, eggshells, claws or paws, and

Submission: Jul 22, 2025 **Revision:** September 5, 2025 **Published date:**September 25 , 2025

ivory are examples of common biological materials.[3] Even while these traditional methods have proven useful, they frequently have drawbacks when samples are degraded, damaged, or lack identifiable morphological characteristics.[4] Consequently, molecular methods like DNA barcoding with mitochondrial genes have become more accurate, repeatable, and dependable methods for identifying species, particularly in wildlife forensic investigations.[5]

A number of characteristics, including its high copy number per cell, lack of recombination, rigid maternal inheritance, heteroplasmy, fluctuating gene expression, and mitotic segregation, make mitochondrial DNA (mtDNA), an extra-nuclear genome, particularly useful in forensic investigations.[6]

The Cytochrome c oxidase subunit I (COI) gene has emerged as the most reliable marker for DNA barcoding among a variety of mitochondrial markers.[7] With the help of a comprehensive worldwide reference database that includes resources like GenBank and the Barcode of Life Data System (BOLD), the COI gene allows for precise identification between closely related species.[8] However, due to a lack of standardized procedures in forensic casework and a lack of reference data for specific species, precise mtDNA species identification remains difficult in India.[9]This study aims to evaluate how well the COI gene identifies animal species in Indian forensic cases with bioinformatics tools for phylogenetic analysis and sequence alignment.

By examining the COI gene sequences of ten Indian species that are commonly found in wildlife crime cases, our study seeks to close these gaps. These species are: *Panthera tigris* (tiger), *Panthera pardus* (leopard), *Elephas maximus* (elephant), *Bos gaurus* (gaur), *Bos taurus* (domestic cow), *Axis axis* (chital), *Axis porcinus* (hog deer), *Macaca mulatta* (rhesus macaque), *Pavo cristatus* (Indian peacock), and *Sus scrofa* (wild boar).These species were chosen to reflect a wider taxonomic range as well as closely related species pairings (such as tiger vs. leopard, gaur vs. cow, and chital vs. hog deer), in addition to their ecological significance and legal protection under India's Wildlife (Protection) Act. A thorough assessment of the COI gene's discriminating ability across various species relatedness levels was made possible by this selection.

The following steps are frequently included in the standard workflow for molecular species identification: (1) DNA isolation from the biological sample; (2) quantification of the extracted DNA; (3) PCR amplification of a specific region of mitochondrial DNA (mtDNA); (4) gel electrophoresis verification of successful amplification; (5) purification of the PCR amplicon; (6) bidirectional sequencing of the purified product; and (7) comparison of the obtained sequence data with reference databases like GenBank or ForCyt.[10]Although species identification also uses additional mitochondrial genes including cytochrome b, 12S rRNA, and 16S rRNA, the COI gene is still the most used and accepted marker for DNA barcoding.

In order to investigate evolutionary links, this study will extract COI sequences from NCBI GenBank[11],validate them using BLAST[12], align them using Clustal Omega[13], and perform pairwise distance analysis and phylogenetic analysis using MEGA12 software[14]. The research's findings are intended to support forensic investigations, create a unified reference for Indian species, and advance the creation of standardized procedures in wildlife forensics. This study emphasizes how crucial molecular-based species identification is to both improving forensic techniques in wildlife crime investigations and conserving India's biodiversity.[15]

Materials and Methods

The present study employed an in-silico methodology to show that, instead of depending on traditional wet-laboratory techniques, species identification and COI gene analysis may be successfully performed using open-access databases and bioinformatics tools[16]. The objective was to demonstrate the reflecting both the potential and existing challenges of applying computational methods in wildlife forensic investigations by offering a non-invasive, cost-effective, and time-efficient substitute for species identification and biodiversity analysis[17]. The full study was carried out in April 2025 over a period of one month.7)

Ten species of great forensic and conservation significance in the Indian context were selected based on their frequent use in genetic studies and documented relevance in forensic casework[18, 19]. These are:*Panthera tigris* (tiger), *Panthera pardus* (leopard), *Elephas maximus* (elephant), *Bos gaurus* (gaur), *Bos taurus* (domestic cow), *Axis axis* (chital), *Axis porcinus* (hog deer), *Macaca mulatta* (rhesus macaque), *Pavo cristatus* (Indian peacock), and *Sus scrofa* (wild boar). In India, these species are commonly found in incidents of illicit poaching, wildlife trafficking, and conservation issues.A common and well tested marker for animal DNA barcoding, the cytochrome c oxidase I (COI) gene was deliberately targeted due to its great inter-species diversity and universality.[7]

Each species' COI gene sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank database.[11]Species-specific keywords (e.g., "Panthera tigris COI gene") were used in a concentrated search. Initial shortlisting of two to three sequences per species was done using certain criteria: high-quality annotation with few ambiguities or unidentified nucleotides; availability of voucher or isolate information; & sequence length (full-length barcodes of roughly 650 bp) [20]. After a thorough analysis, the chosen sequences were downloaded in FASTA (.fasta) format. Only dependable and representative sequences were used for subsequent studies because of this retrieval procedure.

Table 1. A list of specific wildlife species from India that have been selected for in-silico analysis, along with their common names, GenBank accession numbers, and COI gene sequence lengths (in base pairs).

S. No.	Species Name	Common Name	Accession No.	Sequence Length (bp)
1	*Panthera tigris*	Bengal Tiger	MZ099332.1, MZ099331.1	663
2	*Panthera pardus*	Leopard	MZ049429.1, MZ049428.1	658
3	*Elephas maximus*	Indian Elephant	MZ061670.1, MZ061669.1, MZ046731.1	658
4	*Bos taurus*	Domestic Cow	MN714170.1, MZ049020.1, MZ049018.1	16338,658
5	*Bos gaurus*	Gaur	KF808255.1	606
6	*Axis axis*	Chital	KT372098.1, MN226859.1	1545,567
7	*Axis porcinus*	Hog Deer	KF509980.1, KF509978.1, KF509976.1	684,691
8	*Macaca mulatta*	Rhesus Monkey	OR290636.1, OR290633.1, OR290630.1	672,675
9	*Pavo cristatus*	Indian Peacock	LC018112.1, GQ922638.1, GQ922612.1	773,699
10	*Sus scrofa*	Wild Boar	OK244645.1, MT251432.1, KY661881.1	1545,657,697

The Basic Local Alignment Search Tool[12]for nucleotides (BLASTn tool) was used to confirm the species identity upon collection by comparing it to the NCBI nucleotide database. After uploading each FASTA sequence to BLASTn, the highest-scoring hits were assessed using the following criteria: minimum E-value, maximum score, maximum percentage identity, and query coverage. Sequences that demonstrated accurate taxonomic placement and top alignment scores were chosen for additional research. The highest-scoring accession was taken into consideration when the first downloaded sequences did not match the best BLASTn hit. By taking this step, the possibility of dealing with incomplete, poor-quality, or incorrectly recognized sequences was reduced, guaranteeing one high-confidence COI sequence for every target species.

Table 2. BLAST results for each chosen species, including the associated final GenBank accession number, top match species, sequence identity, query coverage, E-value, and maximum score.

S. No.	Species Name	Top BLAST Hit	%Identity	Query coverage	E-value	Max Score	Final Accession No.
1	*Panthera tigris*	*Panthera tigristigris*	100%	100%	0.0	1225	MZ099332.1
2	*Panthera pardus*	*Panthera pardus*	100%	100%	0.0	1216	MZ049428.1
3	*Elephas maximus*	*Elephas maximus*	100%	100%	0.0	1216	MZ061669.1
4	*Bos taurus*	*Bos taurus*	100%	100%	0.0	1216	MN714170.1
5	*Bos gaurus*	*Bos gaurus*	100%	100%	0.0	1120	KF808255.1
6	*Axis axis*	*Axis axis*	100%	100%	0.0	2845	KT372098.1
7	*Axis porcinus*	*Axis porcinus*	100%	100%	0.0	1277	KF509976.1
8	*Macaca mulatta*	*Macaca mulatta*	100%	100%	0.0	1206	OR290633.1
9	*Pavo cristatus*	*Pavo cristatus*	100%	100%	0.0	1291	LC018112.1
10	*Sus scrofa*	*Sus scrofa cristatus*	100%	100%	0.0	1214	OK244645.1

To ensure correct format compatibility with Clustal Omega, the separate FASTA files were combined using Command Prompt operations prior to executing alignments. In addition to a single combined FASTA file with the sequences of all 10 species, separate combined files were created for closely related species pairings (*Panthera tigris* vs. *Panthera pardus*, *Bos gaurus* vs *Bos taurus*, and *Axis axis* vs *Axis porcinus*).

Clustal Omega[13], an online program designed to handle big and complicated biological datasets, was utilized to perform multiple sequence alignment. Guide trees and hidden Markov models are used in the tool's scalable and quick progressive alignment process, which guarantees accuracy and speed. The output was produced in ClustalW format, and the alignment was carried out with the default parameters. Although manual reformatting was necessary to ensure compatibility with MEGA12, Clustal Omega's accuracy in preserving the biological integrity of the sequences was essential for further genetic investigation.

Pairwise distance analysis was carried out using MEGA12[14] for the fine-scale resolution of closely related species pairs. The p-distance model was adopted for the analysis because it is a popular and easy way to calculate genetic distance, and it works best for closely related species with little sequence divergence[21]. Assuming uniform substitution rates, the computation was performed using pairwise deletion of gaps and missing data.*Panthera tigris* vs. *Panthera pardus*, *Axis axis* vs. *Axis porcinus* and *Bos gaurus* vs. *Bos taurus* were the species pairs that were examined. The distance matrices that were produced supported species-level identification in wildlife forensic applications by quantifying the genetic divergence between each pair of species.

Phylogenetic trees were then built using MEGA12 using the aligned datasets. After making the required formatting changes, each file was imported into the program. The Kimura 2-parameter (K2P) model[22], a common model for DNA barcoding research that assumes uniform substitution rates across sites, was used along with the Neighbor-Joining (NJ) method to infer phylogenetic relationships. Particularly, the K2P model was selected over complex ones due to its ability to differentiate between transition and transversion rates, offering a well-balanced method that increases species-level phylogenetic analysis accuracy while preserving computing efficiency. Using a bootstrap approach with 1000 repeats, the branching patterns' reliability was evaluated. The constructed phylogenetic tree gave a visual depiction of the genetic divergences of the chosen species and showed the general evolutionary links between them.

Result and Interpretation

The NCBI GenBank[11] database was used to obtain the cytochrome c oxidase I (COI) gene sequences for the ten chosen species. These sequences were examined using the Basic Local Alignment Search Tool[12](BLASTn) to confirm species identity. With the query value and percentage identity being 100% and the E-value for each sequence being 0.0, the BLAST findings showed that the sequences were extremely accurate and specific to their respective species. These outcomes demonstrated that the sequences that were recovered were genuine and suitable for additional examination.

Sequences that did not exhibit 100% identity or had unclear findings were eliminated in order to guarantee high-quality data. This rigorous quality control procedure made sure that only sequences with high confidence were kept for further examination.

Based on the COI gene sequences of the chosen species, multiple sequence alignments were carried out using Clustal Omega[13] to evaluate the genetic differences between them. The alignment showed a mix of variable areas, which are responsible for interspecies genetic differentiation, and conserved sections, which are crucial for mitochondrial function. The alignment demonstrated the efficiency of the COI gene in species classification[7] by showing that closely related species, such those in the Panthera genus, had significant sequence similarity while more distantly related species showed more divergence. The p-distance model of MEGA12[14] was used to create genetic distance matrices that measured the genetic divergence between species pairings. For the chosen species pairs, the p-distance values were as follows:

Table 3. Genetic distance (p-distance) calculations based on COI gene sequences between pairs of closely related species.

Species 1	Species 2	Genetic Distance (p-distance)
Panthera tigris	*Panthera pardus*	0.0714
Bos taurus	*Bos gaurus*	0.0693
Axis axis	*Axis porcinus*	0.706

Closely related species, like *Bos taurus* vs. *Bos gaurus* and *Panthera tigris* vs. *Panthera pardus*, had relatively low p-distance values because of close genetic relationships. Nonetheless, **there appears to be significant genetic diversity between *Axis axis* (chital) and *Axis porcinus* (hog deer), as indicated by the noticeably high p-distance between these two species. This discovery calls for more research since it suggests either an unexpectedly high evolutionary distance[23] within the Axis genus or possible problems with sequence quality[24].**

The aligned COI gene sequences and the Kimura 2-Parameter[22] (K2P) model with 1000 bootstrap replicates in MEGA12 were used to create a Neighbor-Joining phylogenetic tree. The species were clearly divided into several clades by the tree, which matched their taxonomic identities.

While more distantly related species, like *Bos gaurus* and *Bos taurus*, appeared in separate clades, closely related species, such *Panthera tigris* and *Panthera pardus*, were placed close to one another on the tree. Significantly, **species pairs such as *Axis axis* and *Axis porcinus* were positioned on different branches, which is in accordance with the p-distance analysis's finding of substantial genetic divergence.** The ability of COI sequences to differentiate species across different taxonomic groups was further supported by the formation of individual, well-supported clades with high bootstrap values[25] by other species, such as *Elephas maximus* (elephant), *Sus scrofa* (wild boar), *Macaca mulatta* (rhesus macaque), and *Pavo cristatus* (Indian peacock).

The research validated the usefulness of this gene marker for forensic applications[26] in animal species identification by showing that COI gene sequences may successfully distinguish between closely related and distantly related species.

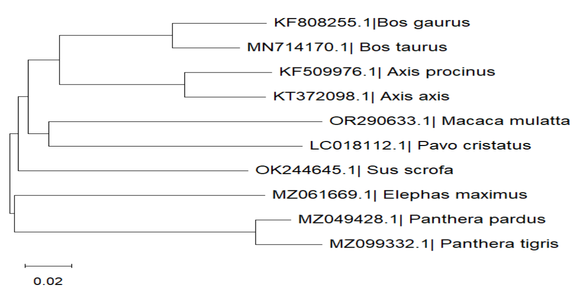

Figure 1: A neighbor-joining phylogenetic tree based on the Kimura 2-Parameter (K2P) model with 1000 bootstrap repetitions in MEGA12 was built utilizing COI gene sequences from ten Indian wildlife species.

Discussion

The efficiency of mitochondrial COI gene sequences in differentiating closely and distantly related Indian wildlife species is confirmed by this in-silico investigation. For all ten taxa, BLAST analysis yielded initial high-confidence species-level identification with 100% query coverage and identity and 0.0 E-values.[11,12] Although BLAST is sufficient for high-quality samples, the limitations in cases of degradation or ambiguity underscore the necessity of additional techniques.[2]

According to Sievers et al., the use of Clustal Omega[13] for multiple sequence alignment increased alignment accuracy, especially when distinguishing closely related species such as *Panthera tigris* and *Panthera pardus*. Additionally, alignment made it possible to detect minute nucleotide variations, which are essential in forensic situations involving deteriorated samples.

Species-level variations were confirmed by genetic distance analysis (p-distance model) of MEGA12[14]. Clear separation was nevertheless possible despite limited divergence between species pairs, such as *Bos gaurus* and *Bos taurus* (0.0693) and *Panthera tigris* and *Panthera pardus* (0.0714). *Axis axis* and *Axis porcinus* have a very high divergence (0.706), which could indicate either major evolutionary separation or inconsistent data. In situations where BLAST is inconclusive, genetic distance analysis bolster forensic evidence.

Strong bootstrap values supported the Neighbor-Joining phylogenetic tree, which was built using the Kimura 2-Parameter[22] model. It clustered species based on their taxonomy and revealed separate clades even among genetically identical species[27]. Phylogenetic trees offer important evolutionary insights, even if they are not always necessary in forensics, particularly in court cases involving endangered species.

The current work expands and improves on earlier studies on the use of DNA barcoding for wildlife identification. The use of the COI gene for species-level identification was first introduced by Hebert et al., although their research was more widely distributed and did not really address the forensic context of Indian wildlife[7]. Ogden et al. established BLAST's usefulness in wildlife forensics, but also acknowledged its limitations for degraded materials[2]. To improve the accuracy of species identification, the current study uses a multi-layered strategy that includes BLAST, multiple sequence alignment, genetic distance analysis, and phylogenetic reconstruction. Verma and Singh looked at the phylogenetics of Indian mammals; however they lacked rigorous sequencing validation and fine-scale resolution[27].

Our work uses strict quality control, choosing only sequences with 100% identity and 0.0 E-values. It also identifies exceptional divergence within the Axis genus, which may indicate a need for additional taxonomic clarification or considerable evolutionary separation. As a result, the study provides methodologically sound, forensically relevant, and region-specific findings that enhance previous methodologies.

The present study has certain limitations. The analysis was based on a limited number of species samples, which may not fully capture the genetic diversity of all possible organisms. Further, the study relied only on the COI gene for species identification, whereas the inclusion of additional markers could improve accuracy and resolution. Another limitation is the dependence on online databases such as BLAST, where the availability and completeness of reference sequences directly affects the results. COI barcoding has limitations, including the potential inability to distinguish hybridizing or closely related species and the potential for errors due to amplifying nuclear pseudogenes (numts) [28, 29]. Additionally, because COI is a mitochondrial marker, its results must be interpreted cautiously, and alternative markers may be needed in complex cases.

However, the study can be extended by increasing the sample size and including more diverse species the findings would be more reliable and widely applicable. Incorporating multiple genetic markers, such as 16S rRNA or cyt b, alongside COI could strengthen species identification and phylogenetic analysis. Developing a local reference DNA barcode database and applying advanced bioinformatics tools or next-generation sequencing can further improve species identification and strengthen forensic applications.

Conclusion

The usefulness of mitochondrial COI gene barcoding as a potent species identification technique in Indian wildlife forensics is further supported by this in-silico study. Clear species difference across ten different taxa was accomplished by the study by using a variety of bioinformatics techniques, such as sequence verification, alignment, phylogenetic analysis, and genetic distance computations. The COI marker's ability to capture genetic distinctiveness is demonstrated by the consistency of results, especially when comparing closely related species.

Crucially, the study shows that whereas quick identification methods perform well in ideal circumstances, complicated forensic situations—like those involving deteriorated samples or closely related species—call for a more sophisticated analytical approach. Methods such as tree-based inference and multiple sequence alignment provide insightful information that enhances the validity and dependability of conclusions based on DNA.

The use of reliable molecular techniques is essential given the surge in wildlife crimes, including poaching and illegal trading. COI barcoding is becoming more and more supported by this type of research as a method of conducting forensic investigations that is both legally and scientifically sound. It is a vital tool for forensic labs and animal conservation organizations due to its affordability, versatility, and connectivity with digital databases.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

Ethical Clearance: Not required as study does not employ live subjects

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