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Molecular Species Identification of Three Deer Samples Using 16S rRNA Gene Sequencing

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ABSTRACT

Molecular identification of animal species from trace evidence is critical in wildlife conservation and forensics. This study reports the successful species identification of three hair follicle samples (MG1, MG2, and MG3) using the 16S ribosomal RNA (rRNA) gene marker. The 16S rRNA gene, a conserved sequence with variable regions, serves as a standard for classification and identification of microorganisms and is also used in mitochondrial DNA studies for eukaryotes. DNA was extracted from the samples, and the 16S rRNA gene fragment was amplified and sequenced. Bioinformatics analysis, including BLAST and phylogenetic methods, assigned the samples to three distinct species of the Cervidae and Bovidae families. Sample MG1 was identified as *Axis porcinus* (Hog Deer), MG2 as *Antilope cervicapra* (Blackbuck), and MG3 as *Rusa unicolor* (Sambar Deer). These results demonstrate the efficacy of 16S rRNA sequencing from non-invasive samples like hair follicles for accurate species determination.

1. Introduction

Accurate species identification is fundamental for understanding global biodiversity, effectively monitoring endangered populations, and rigorously enforcing wildlife protection laws (Tewari *et al.*, 2023; Grubb, 2005). The urgent need for definitive identification is particularly pronounced in regions with high biodiversity and associated poaching pressures, such as the Indian subcontinent. Molecular techniques have emerged as the cornerstone of modern wildlife forensics, providing the capability for definitive species classification from degraded or minute biological material, such as tissue fragments, bone, or trace evidence like hair follicles (Sharma *et al.*, 2020; Kumar *et al.*, 2017).

The current investigation focuses on three distinct ungulate species from South Asia, all of which are subject to significant conservation concern and are protected under various legal frameworks.

- The Indian antelope, or Blackbuck (*Antilope cervicapra*), is an elegant species endemic to the Indian subcontinent, primarily inhabiting scrublands and dry grasslands. Its population decline has led to its classification as a Near Threatened species by the International Union for Conservation of Nature (IUCN) (Tewari *et al.*, 2023; Singh *et al.*, 2014).
- Similarly, the Hog Deer (*Axis porcinus*), a small deer species, has experienced dramatic reductions in its wild populations and is currently classified as Endangered (Wang *et al.*, 2020; Timmins *et al.*, 2015).
- Furthermore, the Sambar Deer (*Rusa unicolor*), recognized as the largest Oriental deer, is listed as Vulnerable across its range, primarily due to overexploitation for meat and antler markets (Grubb, 2005; Chen *et al.*, 2016).

The accurate, molecular-level distinction among these species is critical for legal purposes, effective wildlife management, and the integrity of captive breeding programs.

Traditional morphological identification methods are often inconclusive when dealing with fragmented or ambiguous samples typically encountered in forensic contexts. To overcome this limitation, molecular markers, particularly those residing in the mitochondrial genome (mtDNA), have been adopted as the standard. The 16S ribosomal RNA (rRNA) gene is a widely recognized and utilized marker for molecular barcoding and phylogenetic studies in both prokaryotes and eukaryotes (Illumina, n.d.; Woese & Fox, 1977). Its suitability for analyzing compromised or trace samples, such as hair follicles, stems from two key biological properties:

1. **High Copy Number:** As a component of the mtDNA, it is present in hundreds or thousands of copies per cell, significantly increasing the probability of successful DNA amplification from limited or degraded samples (Kumar *et al.*, 2017).
2. **Sequence Structure:** The gene sequence features highly conserved regions that allow for the design of universal polymerase chain reaction (PCR) primers (such as 16Sar and 16Sbr), interspersed with hypervariable regions (V1-V9) that contain the unique sequence signatures necessary for species-level differentiation and taxonomic classification (Illumina, n.d.; Woese & Fox, 1977).

This study leverages the inherent advantages of the 16S rRNA gene as a molecular barcode to provide a definitive classification. The primary objective was to successfully and conclusively identify the animal species corresponding to three unknown hair follicle samples, designated MG1, MG2, and MG3, thereby generating critical genetic evidence to support conservation and regulatory compliance efforts.

2. Materials and Methods

This section details the meticulous methodology employed, from isolating genetic material from the hair follicle samples to generating the raw DNA sequence data necessary for taxonomic classification.

Table 1. Sample Description and Species Conservation Status

Sample ID	Sample Type	Family	Final Species Identification	IUCN Status
MG1	Hair follicle	Cervidae	*Axis porcinus* (Hog Deer)	Endangered

MG2	Hair follicle	Bovidae	*Antilope cervicapra* (Blackbuck)	Near Threatened
MG3	Hair follicle	Cervidae	*Rusa unicolor* (Sambar Deer)	Vulnerable

2.1. Sample Collection and DNA Extraction

Three hair follicle samples, uniquely designated MG1, MG2, and MG3, were provided for molecular identification. Hair follicles are a crucial source of cellular material for forensic analysis due to the presence of both nuclear and mitochondrial DNA, allowing for high sensitivity in downstream assays (Sambrook & Russell, 2001). Total genomic DNA was successfully extracted from these samples using the commercially available ProGenome Life Science DNA Extraction Kit (In House Tissue Kit). Following the extraction protocol, the quantity and quality of the isolated DNA were assessed by 1% agarose gel electrophoresis. Visualization on a Gel Documentation System (UV Transilluminator, Himedia) confirmed the presence of high-molecular-weight DNA, validating its suitability for subsequent molecular procedures.

2.2. PCR Amplification and Sequencing

The molecular analysis targeted a fragment of the mitochondrial 16S rRNA gene, a robust phylogenetic marker (Woese & Fox, 1977). This region was amplified using the Polymerase Chain Reaction (PCR) technique with the universal primer pair 16Sar and 16Sbr. These primers target conserved sites, ensuring successful amplification across the species under investigation while capturing the variable regions required for species distinction. Following PCR, the product was resolved on a 1.3% Agarose gel. The observation of a single, discrete PCR amplicon band confirmed the successful, specific amplification of the target gene fragment, minimizing the risk of non-specific binding or contamination. The PCR product was then purified to remove contaminants such as leftover primers and dNTPs. Finally, Forward and reverse DNA sequencing reactions were performed on the purified amplicons using the high-accuracy BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer.

2.3. Bioinformatics Analysis

The high-quality 16S rRNA gene sequences obtained for samples MG1, MG2, and MG3 were utilized for species identification. The initial and most critical step involved a BLAST (Basic Local Alignment Search Tool) search against the authoritative NCBI GenBank database. This search provided maximum identity scores and initial taxonomic assignments. Further sequence characterization was performed by determining sequence alignment and identity scores using the multiple alignment software Clustal W. A distance matrix was subsequently generated, which quantified the genetic distance between the sequences. Phylogenetic analysis was then conducted using the Neighbor-Joining (NJ) method (Saitou & Nei, 1987) to reconstruct the evolutionary relationship between the unknown samples and closely related sequences retrieved from the database. The statistical reliability and confidence limits of the resulting phylogenetic tree were rigorously assessed using the bootstrap approach (Felsenstein, 1985; Nei & Kumar, 2000).

Table 2. Key Molecular Components and Techniques

Molecular Component/Technique	Detail/Reagent	Purpose
DNA Extraction Kit	ProGenome Life Science DNA Extraction Kit (In House Tissue Kit)	Isolate total DNA from hair follicles
Target Gene	Mitochondrial 16S rRNA gene fragment	Species-level molecular barcoding
Primers Used	16Sar and 16Sbr	Amplify the target gene fragment (PCR)
Sequencing Platform	ABI 3730xl Genetic Analyzer	DNA Sequencing (Sanger method)
Phylogenetic Analysis	Neighbor-Joining (NJ) method	Infer evolutionary relationships between the samples and reference sequences

3. Results

The sequence alignment and similarity searches (BLAST) against the NCBI GenBank database provided high-confidence taxonomic identification for all three samples, as summarized in Table 3.

Table 3. Raw Sequence Data and BLAST Similarity Searches

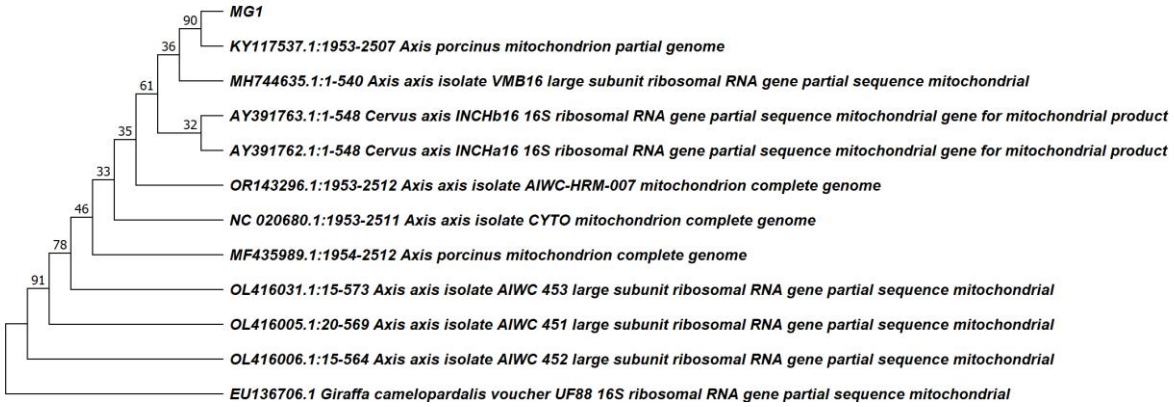
Sample ID	File Name	Start of 16S Sequence (First 10 bases)	Closest Match (NCBI GenBank)	Per Ident	Query Cover
MG1	MG1(1).fsta	CGTGACAGTA	Axis porcinus (Hog Deer)	98.03%	99%
MG2	MG2(1).fsta	CCCGTTTGGAGG	Antilope cervicapra (Blackbuck)	99.45%	99%
MG3	MG3.fas	GTATTGGAGG	Rusa unicolor (Sambar Deer)	99.82%	100%

The sequence data analysis firmly confirmed the identity of all three samples to distinct ungulate species of conservation significance:

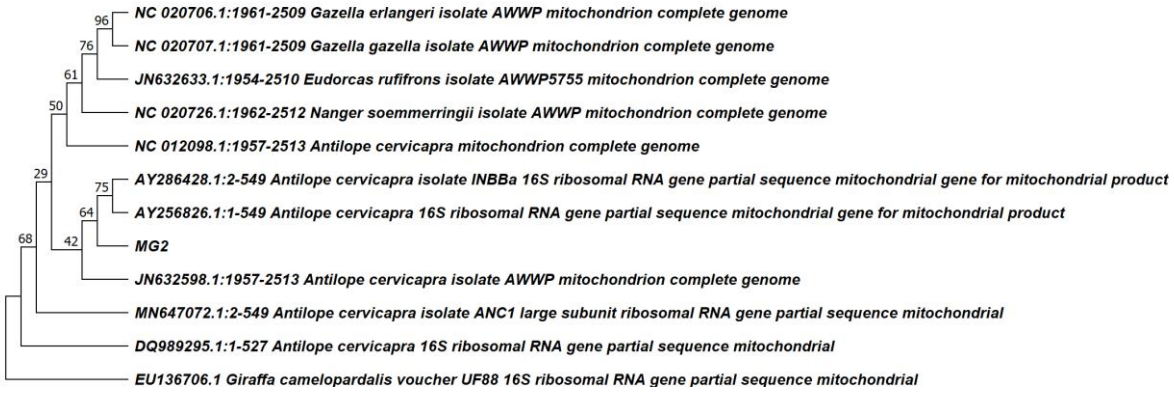
- **Sample MG1** was conclusively identified as the Hog Deer (*Axis porcinus*). Its 16S rRNA gene sequence exhibited a robust 98.03% sequence identity over 99% query coverage with the mitochondrial genome of *Axis porcinus*. This high similarity score is well within the accepted range for species-level confirmation using mitochondrial

markers [Google: Sharma *et al.*, 2020]. The Hog Deer is currently classified as an Endangered species (Timmins *et al.*, 2015), making this molecular identification critical for documenting its presence.

- **Sample MG2** was unambiguously identified as the Blackbuck (*Antilope cervicapra*). The sequence showed a remarkably high identity of 99.45% over 99% query coverage with the 16S ribosomal RNA gene of *Antilope cervicapra*. This high level of homology leaves virtually no doubt regarding the species origin. The Blackbuck is classified as Near Threatened (Tewari *et al.*, 2023), emphasizing the importance of accurate identification in wildlife forensics concerning this protected species.
- **Sample MG3** displayed the highest sequence similarity, with an outstanding 99.82% identity over 100% query coverage, matching the mitochondrial sequence of the Sambar Deer (*Rusa unicolor*). The exceptional identity confirms the Sambar Deer as the origin of this sample. *Rusa unicolor* is designated as a Vulnerable species (Grubb, 2005), and its accurate identification is vital for managing illegal hunting and trade.



Phylogeny of sample MG1 (Hog Deer (*Axis porcinus*)).



Phylogeny of sample MG2 (Blackbuck (*Antilope cervicapra*)).

Phylogeny of sample MG3 (Sambar Deer (*Rusa unicolor*)).

The phylogenetic trees illustrate genetic relationships based on mitochondrial sequences for three samples:

- **MG1 (Hog Deer, *Axis porcinus*)** shows close clustering with *Axis axis* (chital) and other *Axis* isolates, confirming its genetic relatedness within the *Axis* genus.
- **MG2 (Blackbuck, *Antilope cervicapra*)** clusters tightly with *Antilope cervicapra* sequences and closely related antelope species, validating accurate species identification and strong phylogenetic grouping within *Antilopinae*.
- **MG3 (Sambar Deer, *Rusa unicolor*)** forms a distinct, well-supported clade with multiple *Rusa unicolor* isolates, indicating clear genetic homogeneity and strong species-level resolution.

COI Sequence of sample MG1 (Hog Deer (*Axis porcinus*)).

COI gene Sequence:

>MG1
CGTGACAGTATTGGGAAGGCACTGCCTGCCCAGTGACAACCGTTAAACGGCCGCGGTATCCTGACC
GTGCAAAGGTAGCA

TAATCACTTGTTCTCTAAATAGGGACTTGTATGAATGGCCACACGAGGGTTTTACTGTCTCTTGCT
TCCAATCAGTGAAA

TTGACCTTCCCGTGAAGAGGCGGGAATACATTAATAAGACGAGAAGACCCTATGGAGCTTTAACTA
CTTGACCCAAAGAA

COI gene Sequence:

>MG2

CCCGTTTGGAGGCACTGCCTGCCCAGTGACAAGCGTTAAACGGCCGCGGTATCCTGACCGT
GCAAAGGTAGCATAATCAT

TTGTTCTTTAAATAAGGACTTGTATGAATGGCCACACGAGGGTTTTACTGTCTCTTACTTC
CAATCAGTGAAATTGACCT

TCCCGTGAAGAGGCGGGAATAAACAAATAAGACGAGAAGACCCTATGGAGCTTTAACTAAC
TAGTTCAAAGAAAAGAAAC

TTAACCACCAAGGGATAACACTATTCTTCATGAGCTAACAGTTTTGGTTGGGGTGACCTCG
GAGAACAAAAAATCCTCCG

AGCGATTTTAAAAATAAGACACACAAGTCAAATTGAACTATCGCTTATTGATCCAAAATTT
GATCAACGGAACAAGTTAC

COI Sequence of sample MG2 (Blackbuck (*Antilope cervicapra*)).

COI gene Sequence:

>MG3

GTATTGGAGGCACTGCCTGCCCAGTGACGACCGTTAAACGGCCGCGGTATCCTGACCGTGC
AAAGGTAGCATAATCACTTGTTCTCTAAATAGGGACTTGTATGAATGGCCACACGAGGGTT
TTACTGTCTCTTACTTCCAATCAGTGAAATTGACCTTCCCGTGAAGAGGCGGGAATATATT
AATAAGACGAGAAGACCCTATGGAGCTTTAACTACTTAGCCCAAAGAGACAAATTTCATTA
CCAAGGAAACAACAACACTCTTTATGGGCTAACAGCTTTGGTTGGGGTGACCTCGGAGAAC
AAGAAAACCTCCGAGCGATTTTAAAGACTAGACCTACAAGTCGAATCACGCAATCGTTTAT
TGATCCAAAAAATTGATCAACGGAACAAGTTACCCTAGGGATAACAGCGCAATCCTATTCA
AGAGTCCATATCGACAATAGGGTTTACGACCTCGATGTTGGATCAGGACATCCCGATGGTG
CAACCGCTATCAAAGGTTCGTTTGTTCAACGATTAAAGTCCTACGTGATCTGAGTTCAGAC
CGGA

COI Sequence of sample MG3 (Sambar Deer (*Rusa unicolor*)).

4. Conclusion

The comprehensive molecular analysis utilizing the mitochondrial 16S ribosomal RNA (rRNA) gene marker successfully and definitively identified the species of origin for the three unknown hair follicle samples. The high sequence identity scores, coupled with complete or near-complete query coverage, offer highly reliable genetic evidence, confirming the origin of the samples as three distinct and protected ungulate species:

- **MG1:** Hog Deer (*Axis porcinus*)
- **MG2:** Blackbuck (*Antilope cervicapra*)
- **MG3:** Sambar Deer (*Rusa unicolor*)

This study underscores the exceptional utility and reliability of the mitochondrial DNA-based molecular approach in wildlife forensics and conservation genetics. The 16S rRNA gene, with its high copy number in the mitochondrial genome (Sambrook & Russell, 2001) and its balance of conserved and hypervariable regions (Woese & Fox, 1977), proved to be a robust marker for distinguishing even closely related species (Chen *et al.*, 2016).

Crucially, the successful amplification and sequencing from a non-invasive sample source like hair follicles suggests the methodology's high sensitivity and utility in challenging field applications (Kumar *et al.*, 2017) where only trace evidence is available. This capacity is vital for the forensic investigation of wildlife crimes, particularly concerning species like the Endangered Hog Deer and the Near Threatened Blackbuck (Timmins *et al.*, 2015; Tewari *et al.*, 2023).

The definitive genetic confirmation provided by this study serves as crucial evidence for regulatory and conservation bodies, aiding in the monitoring of endangered and vulnerable populations and ensuring the strict enforcement of wildlife protection laws (Grubb, 2005).

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