Molecular identification of Indian Peafowl *Pavo cristatus* Linnaeus, 1758 (Aves: Phasianidae) through DNA Barcoding using feather calamus

Comment [B1]: (*Pavo cristatus*)

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ABSTRACT

The Indian Peafowl Pavo cristatusLinnaeus, 1758, the national bird of India, is a member of the Phasianidae family, known for its extravagant feather ornaments and cultural importance. Accurate identification of this species is crucial for effective conservation strategies, especially in regions where it faces threats from habitat loss and poaching. This study employs DNA barcoding techniques to achieve molecular identification of the Indian Peafowl using shed feathers as non-invasive samples. Mitochondrial cytochrome c oxidase subunit I (COI) gene sequences were analyzed to develop a reliable DNA barcode for the species. The study successfully demonstrates that DNA extracted from shed feathers can produce high-quality barcodes, enabling precise species identification. This method offers a valuable tool for wildlife monitoring and biodiversity assessments, facilitating the conservation efforts for birds by providing an efficient and non-intrusive means of tracking its population and distribution. Our findings highlight the potential of DNA barcoding in enhancing the accuracy of species identification in avian studies, contributing to the broader field of molecular ecology.

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KeywordsDNA barcoding; Molecular markers; Species identification; Wildlife; Forensics; Animal taxonomy; Biodiversity.

1. INTRODUCTION

Anthropogenic activities have deeply impacted the natural world, leading to significant changes in ecosystems and the biodiversity. Industrialization, urbanization, and agricultural expansion have drastically altered landscapes, resulting in habitat destruction and fragmentation [1]. These changes are compounded by pollution, climate change, and the introduction of invasive species, all of which exert additional pressures on native wildlife populations [2]. Among these activities, illegal poaching stands out as a particularly severe threat to biodiversity. Poaching for bushmeat, feather, traditional medicine, and the illegal wildlife trade has driven numerous species to the brink of extinction [3]. High-value targets, such as elephants for ivory, rhinoceroses for their horns, and tigers for their pelts, birds for their beautiful feathers, which are used in fashion and decoration, but also for their meat, which is a staple in many diets, and their oil, which has various culinary and medicinal applications, are not the only victims; many lesser-known species are also at risk due to the lucrative market for exotic pets and animal parts [4].

Morphologically distinguishable taxa may not require DNA barcoding for species level identification; however, subspecies, cultivars, morphotypes, mutants, species complexes, and clones can be diagnosed with molecular barcoding [5]. DNA barcoding has also been applied to wildlife forensic cases where only animal body parts are available for species identification, without requiring the whole animal body [6,7,8]. Illegal hunting is one of the major threats to many animal groups, with estimates suggesting that illegal hunting kills

millions of vertebrates annually [3]. In suspected cases of illegal hunting, the only evidence available may be pieces of meat, skin, bone, feathers or other unused animal parts. In such cases, species identification can only be dependably determined using molecular technologies[9,10], as much of the morphological characteristics are missing. The Consortium for the Barcode of Life (CBOL), an international initiative dedicated to the use of DNA barcoding, promotes a tool for species identification based on a single standard DNA marker: a fragment of the COI mitochondrial DNA gene, used as a global standard for species identification[11]. Generated sequences are matched to reference sequences in the National Center for Biotechnology Information (NCBI) and the Barcode of Life Data Systems (BOLD) databases to confirm the species identification.

1.1. Taxonomic and Morphologic Status of the Indian Peafowl

The Indian Peafowl (*Pavo cristatus* Linnaeus, 1758), the national bird of India belonging to the family Phasianidae, is one of the most recognizable avian species due to its vibrant plumage and elaborate courtship displays. This species is native to the Indian subcontinent and has been introduced to various parts of the world. Morphologically, the Indian Peafowl exhibits pronounced sexual dimorphism: males (Peacocks) possess an iridescent blue neck and breast, and an iconic train of elongated upper tail coverts adorned with eye-like patterns, whereas females (Peahens) display more muted brownish colors, aiding in camouflage during nesting [12]. Despite the distinct morphological features that aid in the visual identification of the Indian Peafowl, there are subspecies and morphotypes within the species that may exhibit subtle variations. Additionally, hybridization with other peafowl species in captivity can further complicate morphological identification[13]. Moreover, only specimen samples such as feathers or other body parts obtained from suspected poaching sites pose difficulties in species-level identification, necessitating the use of DNA barcoding to facilitate wildlife forensic studies.

1.2. Necessity of DNA Barcoding Using Feathers in Avian species identification

The application of DNA barcoding has emerged as a critical tool for accurate species identification, particularly in wildlife forensics and conservation biology [5]. DNA barcoding involves the analysis of a standardized region of the mitochondrial cytochrome c oxidase subunit I (COI) gene, providing a unique genetic signature for species identification [5]. This technique is especially valuable for identifying species from non-invasive samples such as shed feathers, which can be collected without disturbing the animal. DNA barcoding using shed feathers has emerged as a crucial tool for avian species identification, enabling precise and non-invasive sampling of genetic material. This technique utilizes short, standardized gene regions, such as the cytochrome c oxidase I (COI) gene, to differentiate species based on their unique genetic sequences. Unlike traditional methods that require capturing and handling birds, DNA barcoding from feathers allows researchers to collect samples with minimal stress to the animals, which is particularly valuable for studying endangered or elusive species. Moreover, this method facilitates large-scale ecological and biodiversity studies by providing a reliable means to monitor bird populations and track migratory patterns also. Consequently, species identification from feathers, especially when collected from poaching sites, requires the adoption of DNA barcoding to enhance our understanding of avian biodiversity and support conservation efforts globally.

2. MATERIAL AND METHODS

2.1. Sample collection, DNA extraction and PCR amplification

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The feather sample was collected from Kathlour Wildlife Sanctuary, located in Pathankot, Punjab, India (32.268278 N, 75.447637 E). This collection was part of a faunal survey conducted from 21-29 December 2021, aimed at studying the fauna of some conservation areas in Punjab i.e. Rakh Sarai Amanat Khan, Ranjit Sagar, and the Beas River. The survey focused on documenting the biodiversity within these regions to inform and enhance conservation efforts. During the faunal survey on 23rd December 2021, a few feather samples were collected from a suspected poaching site.

The genomic DNA was extracted from the feathers by using Qiagen DNA easy blood and tissue kit, following the manufacturer's protocols which involves a streamlined process designed for high yield and purity. First, the feather's calamus (quill) is carefully cleaned and cut into small pieces. These pieces are then incubated in a lysis buffer (Buffer ATL) with proteinase K, which digests proteins and releases DNA from the feather tissues. This mixture is incubated at 56°C until the feather calamus is completely lysed, usually for several hours or overnight. Following lysis, the sample is mixed with Buffer AL and ethanol, promoting DNA binding to the silica membrane of the spin column provided in the kit. The mixture is then transferred to the spin column and centrifuged, allowing the DNA to adhere to the column while impurities pass through. The column is washed several times with Buffer AW1 and Buffer AW2 to remove any remaining contaminants. Finally, the DNA is eluted from the column using Buffer AE or water, ensuring the recovery of purified genomic DNA. This DNA is then ready for use in various downstream applications, such as PCR amplification and sequencing, with the Qiagen kit providing a reliable and efficient extraction method.

Extracted DNA was quantified on agarose gel electrophoresis using a genomic ladder (GelPilot® 100 bp Plus). DNA thus obtained was subjected to PCR amplification using Eppendorf, Master Cycler. Each PCR reaction of 50 µL consisted of 5 µL10X Qiagen master mix, 2 µL of 10 mMdNTP mix, 1 µL (20 pmol/µL) each of gene-specific forward and reverse mt COI primers (BirdF1, Fwd_seq: TTCTCCAACCACAAAGACATTGGCAC, BirdR1, Rev_seq: ACGTGGGAGATAATTCCAAATCCTG), 0.5 µL Dream Taq DNA polymerase (5 U/µL), 5 µL DNA (50 ng/µL), and 35.5µL sterile water. Thermo-cycling parameters used for the study consisted of an initial denaturation of 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at specific temperatures for 1 minute, extension at 72°C for 1 minute. PCR amplification was thoroughly monitored by the inclusion of a positive test sample (sample that has shown amplification in the past PCR attempts) and also with a negative test sample. After the amplification PCR products was stored at 4°C. The amplified products were analyzed on 1.5% agarose gel electrophoresis. The resultant PCR amplified products were cleaned up by using Qiagen'sQIAquick® PCR Purification Kit and subjected to DNA sequencing by using Applied Biosystems 3500 Genetic Analyzer using BigDye 3.1 sequencing kit (Applied Biosystem&Eurofin genomics, Bangalore). Each specimen PCR sample was bi-directionally sequenced and checked for homology, insertions and deletions, stop codons, and frameshifts.

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3. RESULTS AND DISCUSSION

3.1. DNA polymorphism analysis

These sequences were aligned along with additional mitochondrial COI sequences retrieved from the NCBI, GenBank, and the sequence generated from the present study with Chromas (Version 2.6.6) and MEGA Version 11[14]. Based on similarity search the generated COI sequences showed similarity as Pavo cristatus and were then deposited in NCBI GenBank database and accession number was obtained (ON527520). The identification of species was confirmed by available feather morphology methods and also by using the BLAST

program, NCBI [15]. The sequence was then used for polymorphism studies and further analysis with the COI sequences deposited from Pakistan, Vietnam, China, etc. based on the geographical distribution and also as per available sequences from the NCBI nucleotide database.

The molecular phylogenetic analysis of the Bird feather calamus sample sequence was also conducted using the Neighbor-Joining method on the mitochondrial cytochrome c oxidase I (COI) gene. This gene, commonly employed in DNA barcoding, was extracted from the feather calamus of the Indian Peafowl. The Neighbor-Joining method, a widely used technique for constructing phylogenetic trees, was applied to infer evolutionary relationships based on the genetic distances among sequences. The analysis revealed a clear phylogenetic placement of the Indian Peafowl within the Phasianidae family, confirming its close genetic relationships with other species in the genus Pavo. The resulting phylogenetic tree showcased well-supported branches, indicating robust clustering of the Indian Peafowl sequences with their closest relatives. This molecular phylogenetic approach provided insightful data on the evolutionary lineage of *Pavo cristatus*, demonstrating the effectiveness of using mitochondrial COI gene sequences from feather calamus in DNA barcoding studies.

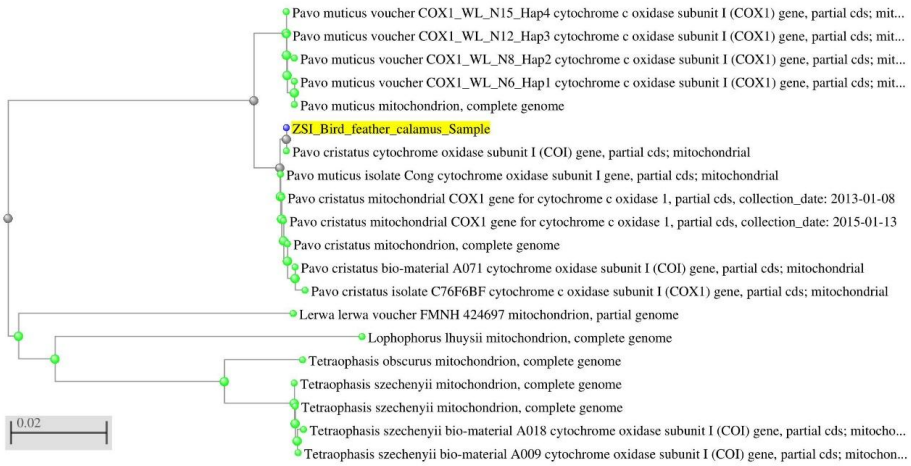

Figure.1. MolecularPhylogenetic analysis by Neighbour Joining method using mitochondrial cytochrome c oxidase 1 gene of Indian Peafowl(*Pavo cristatus*Linnaeus, 1758) through DNA Barcoding using feather calamus

Additionally, NCBI MSA Viewer 1.25.0 is a powerful tool for analyzing multiple sequence alignments, facilitating detailed comparative analysis of genetic sequences. In the present study, the *Pavo cristatus*(Indian Peafowl) sequence was identified using the BLAST (Basic Local Alignment Search Tool) algorithm, which compares the queried sequence against a database of known sequences to find regions of similarity. The MSA Viewer was then employed to visualize and interpret the multiple sequence alignment results. This tool employes to align the query sequence (ZSI Bird feather calamus sample sequence) with those of other related species, highlighting conserved regions, identifying genetic variations, and inferring evolutionary relationships. The visual representation provided by the MSA Viewer aids in the clear identification of sequence homologies and differences, making it an essential resource for molecular biologists studying the genetic makeup and evolutionary history of the Indian Peafowl.

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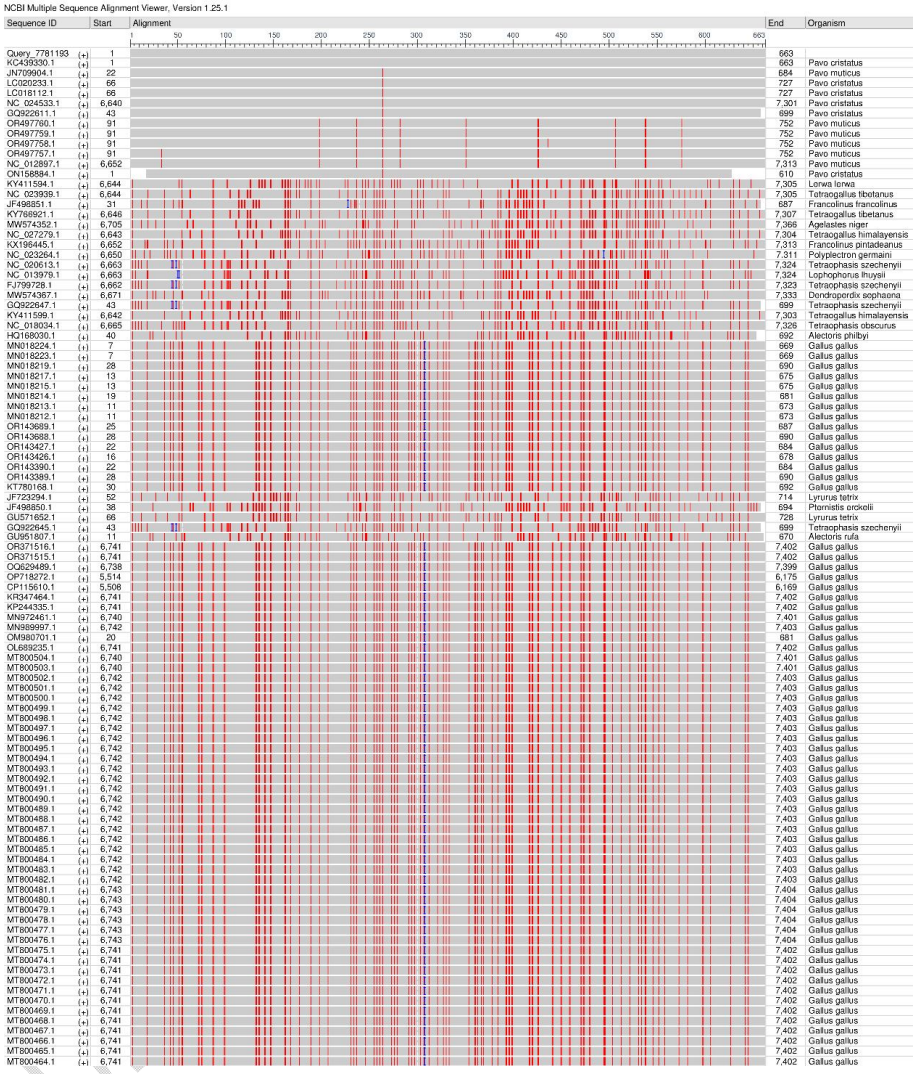

Figure.2. NCBI Multiple Sequence Alignment Viewer 1.25.0 results showing database of known sequences regions of similarity

4. DISCUSSION

The loss of biodiversity due to human-induced pressures has far-reaching consequences. Ecosystems rely on a delicate balance of species interactions, and the removal of even a single species can disrupt ecological networks, leading to cascading effects that can alter entire ecosystems [16]. Biodiversity loss diminishes ecosystem resilience, reducing the ability of ecosystems to provide essential services such as pollination, water purification, and carbon sequestration, which are vital for human well-being [17]. As we face this biodiversity

crisis, it is imperative to develop and implement effective conservation strategies. These must include measures to curb illegal poaching and trade, protect and restore habitats, and promote sustainable practices that harmonize human activities with the needs of the natural world [18]. Conservation efforts should include accurate species identification and population monitoring, tools that are essential for understanding and mitigating the impacts of human activities on biodiversity [5].

The identification of bird species from shed feathers has become increasingly accurate through DNA analysis techniques. Feather calamus/barbs have proven to be a reliable source of mitochondrial DNA (mtDNA), which allows for precise species identification even from minimal and degraded samples. This approach is particularly valuable in forensic wildlife investigations where the integrity of the feather needs to be maintained for other analyses. Studies have shown that mtDNA can be successfully extracted and sequenced from just a few feather calamus, making this a minimally destructive method that preserves the physical attributes of the sample for further morphological studies[19]. Moreover, advancements in DNA barcoding, which involves the use of a specific mitochondrial gene (COI), have further enhanced the accuracy of species identification by distinguishing between closely related species and identifying potential hybridization events [5]. This methodology is essential for applications in conservation biology, archaeology, and forensic science, where accurate species identification is crucial [20].

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5. CONCLUSION

The study demonstrates the efficacy of using DNA barcoding techniques for the molecular identification of the Indian Peafowl through non-invasive samples such as shed feathers. This method not only ensures accurate species identification but also provides a valuable tool for wildlife monitoring, biodiversity assessments, and conservation efforts. The successful application of DNA barcoding in this study highlights its potential to enhance species identification accuracy in avian studies, thereby contributing significantly to the field of molecular ecology.

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