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Identification of Indian Peafowl *Pavo cristatus* Linnaeus, 1758 (Aves: Phasianidae) Using Feather Calamus by Molecular Genetic Method

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Authors' contributions

This work was carried out in collaboration among all authors. Author VMSK designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AK and MCP managed the analyses of the study, and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The ornate and vibrant bird, *Pavo cristatus* Linnaeus, 1758 belongs to Phasianidae family and is proud to be the national bird of India due to it is extravagant feathers and cultural significance. Thus, the accurate identification of this species is significant for its conservation as well as for areas where it is endangered due to factors such as habitat destruction and hunting. This study applies molecular analysis for the identification of the Indian peafowl by analyzing shed feather samples

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non-invasively through DNA barcoding. Barcoding the species was done by using the mitochondrial cytochrome c oxidase subunit I (COI) gene sequences. The study is rightly able to establish that DNA obtained from shed feathers can generate DNA barcodes, which help with species identification. This method provides a useful instrument for the wildlife tracking and the conservation of birds by providing an efficient and non-harmful way of identifying and tracking the population of the species. These results show that DNA barcoding could be useful in increasing the efficiency of species identification, differentiation in avian research to the broader field of molecular ecology for the monitoring, conservation and management.

Keywords: DNA barcoding; molecular markers; species identification; wildlife; forensics.

1. INTRODUCTION

Human activities have greatly influenced the environment in a negative way thus affecting natural systems and the biological diversity [1]. These are further augmented by pollution, climate change, and the addition of new species that create further stress on native wildlife [2]. Of these activities, poaching can be considered the most significant threat to the species' diversity. Hunting for meat, feather, traditional medicine and the black-market trade has pushed many species to near extinction [3,4,5]. Targets of such high value include elephants for ivory, rhinoceroses for horns, tigers for their pelt and birds for their fine feathers [6].

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 [7]. 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 [8,9,10]. 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 [11,12], 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 (mtDNA) gene, used as a global standard for species identification [13]. 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

Indian peafowl or taxonomically known as *Pavo cristatus* Linnaeus, 1758 is one of the most familiar birds belonging to Phasianidae family and is the national bird of India. This species is endogenous to the Indian subcontinent but has been acclimatized in several regions of the globe. Morphologically, the Indian peafowl exhibits pronounced sexual dimorphism: males (peacocks) have an iridescent blue neck and breast and a long train of elongated upper tail coverts with eyelike shapes on them unlike female peahens which are brown in color and help them blend in the ground especially when they are laying [14]. However, the Indian peafowl, though apparently easily distinguishable due to its morphological characteristics, may have some varieties and forms that are not easily distinguishable from each other. Besides, interbreeding with other species of peafowl in enclosures also makes morphological identification even more challenging [15]. However, samples like specimens, feathers or any other body part collected from the suspected poaching areas present challenges in species identification at the species level, necessitating the use of DNA barcoding to facilitate wildlife forensic studies.

1.2 Necessity of DNA Barcoding Using Feathers in Avian Species Identification

Species identification using DNA barcoding has become standard in the identification of species, especially in the area of wildlife forensics as well as the area of conservation [7]. DNA barcoding uses a specific region of the mitochondrial COI

gene to create a genetic signature for species [7]. This technique is particularly useful for species identification from samples that can be collected without affecting the animal such as feathers, which may have shed naturally. Shed feather DNA studies has become one of the most important techniques in DNA barcoding as it is accurate, non-invasive, and can be easily applied to avian species. This technique uses small, particular gene segments, for example, COI gene, which distinguishes species in terms of genetic differences. Compared to other approaches that involve trapping and disturbing birds, DNA barcoding from feathers enables the researchers to obtain samples without physically capturing the animals and often disturbing them, which is especially helpful for species that are threatened or difficult to find. Furthermore, this method enables ecological and biodiversity surveys on a massive scale because it is an effective way of monitoring bird populations and migration as well. Therefore, identification of species from feathers particularly those collected from poaching sites can only be done by developing the application of DNA barcoding to increase the knowledge on bird diversity and their conservation across the world.

2. MATERIALS AND METHODS

2.1 Sample Collection, DNA Extraction and PCR Amplification

The feather sample was taken from Kathlour Wildlife Sanctuary situated in Pathankot, Punjab, India at a geographical co-ordinate of 32. 268278 N, 75. 447637 E. This sample collection was part of a faunal survey done from 21st to 29th December 2021 to find about the faunal diversity of some of the conserved areas of Punjab, i.e. Rakh Sarai Amanat Khan, Ranjit Sagar, and the Beas River. The survey was conducted with an aim of recording the levels of diversification within these areas, in order to improve on the trends in the conservation efforts. On 23rd December 2021 while conducting faunal survey, few feather samples were picked up from a suspected poaching ground and were used in the present study.

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 or the main body is shaved and sliced into small portions. These pieces are then mixed with a lysis buffer (Buffer ATL) containing proteinase K which breaks down the proteins and frees the DNA from the feather tissues. This mixture is then incubated at 56°C till the feather calamus is completely digested, which usually takes a few hours to overnight. After lysis, the sample is mixed with buffer AL and ethanol and DNA binds to the silica membrane of the spin column included in the kit. The mixture is then applied to the spin column and spun to capture the DNA to the column while the rest of the debris is washed through. The column is then washed with buffer AW1 and then with buffer AW2 several times to eliminate any contaminants. Last but not the least, the DNA is washed off the column with buffer AE or molecular grade water to obtain pure genomic DNA. This DNA is then ready for use in a number of downstream applications which include PCR amplification and sequencing and the Qiagen kit was observed to be efficient in the extraction process.

The presence of extracted DNA was estimated on 1% agarose gel and employing a DNA molecular weight marker (GelPilot® 100 bp Plus). DNA thus obtained was then amplified through PCR 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 conditions used in the present study include a denaturation step of 5min at 94°C, and 30 PCR cycles of 1min at 94°C, annealing at particular temperature for 1 min and extension for 1 min at 72°C. The PCR amplification was closely observed by the use of a positive test sample as well as a negative test sample. The amplification PCR products was then stored at 4°C. The amplified products were characterized on 1. 5% agarose gel electrophoresis. The PCR amplified products were then purified with QIAquick® PCR Purification Kit of Qiagen to remove the unincorporated nucleotides and the DNA samples were then sequenced with the help of Genetic Analyzer of Applied Biosystems 3500 using BigDye 3. 1 sequencing kit (Applied Biosystem & Eurofin genomics, Bangalore). All the PCR samples of the specimen were bidirectional sequenced, and homology check,

insertion and deletion, stop codon, frame shift was also done.

3. RESULTS AND DISCUSSION

3.1 DNA Polymorphism Analysis

These sequences obtained in the current study aligned with Chromas (Version 2. 6. 6) and MEGA Version 11 [16] and also were imported along with other available mitochondrial COI sequences obtained from NCBI, GenBank. From similarity search the generated COI sequences was resembled to *Pavo cristatus* and was submitted to NCBI GenBank database and accession number was assigned (ON527520). The identification of species was confirmed by the feather morphology that is available in the literature as well as by using the BLAST program, NCBI [17]. The obtained sequence was then used for polymorphism studies and additional analysis with the COI sequences posted from Pakistan, Vietnam, China and others based upon geographical distribution and also as per the sequences available at the NCBI nucleotide database.

The molecular phylogenetic analysis of the bird feather calamus sample sequence was also carried out using the Neighbor-Joining method on the mitochondrial COI gene. This gene, typically used in DNA barcoding, was obtained from the feather calamus of the Indian peafowl. The neighbor-joining method that is widely used in constructing the phylogenetic trees was used to analyze the evolutionary relationships using genetic distances between the sequences. The study showed a precise phylogenetic position of Indian peafowl in the Phasianidae family and common genetic relations to other *Pavo* genus species. The generated phylogenetic tree revealed highly significant branch points and proper grouping of the Indian peafowl sequences with their nearest kin. This molecular phylogenetic study proved useful in generating data on the evolutionary history of *Pavo cristatus* and highlighted the application of mtCOI gene sequences of feather calamus in DNA barcoding studies.

Additionally, NCBI MSA Viewer 1.25.0 is an effective program for dealing with multiple alignment of sequences, which allows for further comparison of genetic sequences. In the present study, the *Pavo cristatus* sequence was searched with the help of Basic Local Alignment Search Tool (BLAST) which compares a given sequence with the other known sequences to find out the similarity. The MSA Viewer was then used to analyse the multiple sequence alignment outcomes. This tool used to compare the query sequence (ZSI bird feather calamus sample sequence) with other reference species' sequences to find the similarities, differences, and to understand the evolutionary relationships. The visualization tools in the form of MSA Viewer help in the proper demarcation of the sequence similarities and dissimilarities both of which play a vital role in the identification and characterization of the genetic profile and evolution of the Indian peafowl.

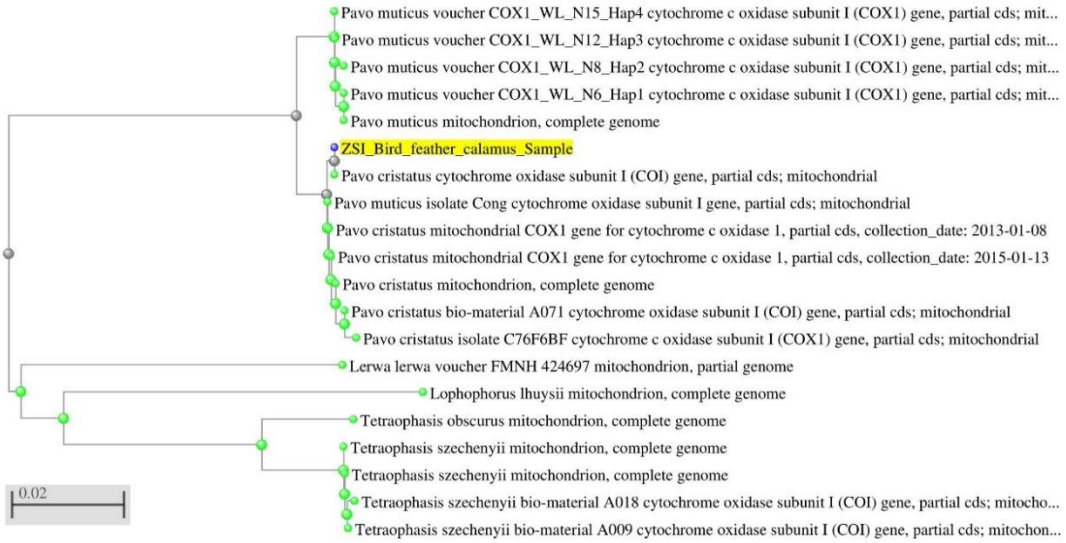

Fig. 1. Molecular phylogenetic analysis by neighbour joining method using mitochondrial cytochrome c oxidase 1 gene of Indian peafowl through DNA Barcoding using feather calamus

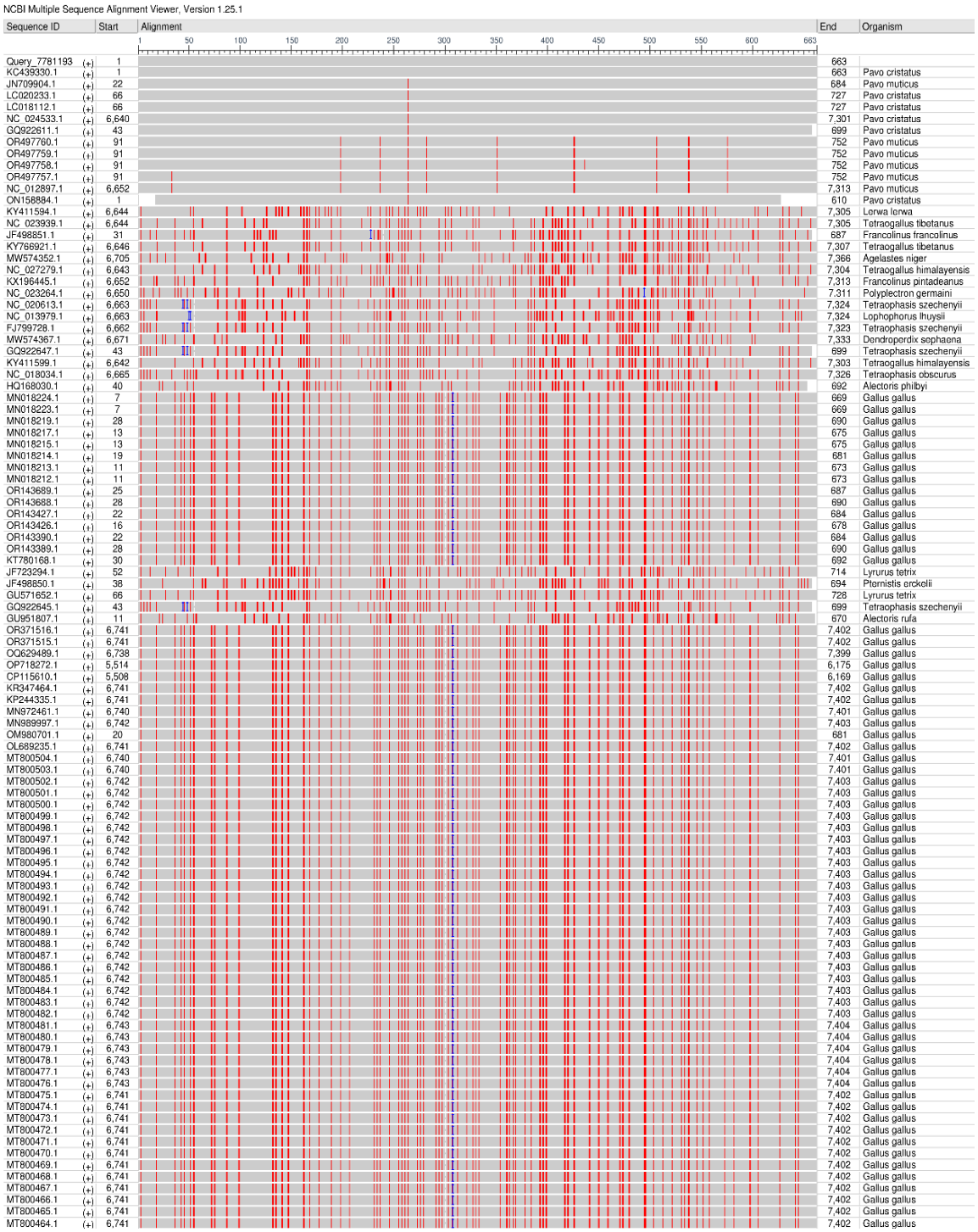

Fig. 2. NCBI Multiple Sequence Alignment Viewer 1.25.0 results showing database of known sequences regions of similarity

3.2 Discussion

Loss of biodiversity as a result of human induced pressures has implications for the natural system. Real ecosystems are complex and interconnected, and any extraction of one species will have an impact on the ecosystems' web and can change the ecosystem [18]. Deforestation decreases the capacity of ecosystems to support the productivity of vital ecosystem services like pollination, water purification, and carbon sequestration which are

beneficial to human beings [19]. However, there is need to come up with and put into practice effective measures in dealing with this biodiversity crisis. These should consist of strategies to combat unlawful hunting and selling, preserve and rehabilitate ecosystems, and encourage the integration of mankind with the environment in a responsible manner [20]. Conservation measures should involve species identification and population estimates; two crucial processes in management of effects resulting from human activities on the biota [7].

Through the molecular methods of DNA analysis, the identification of the bird species from shed feathers has been made much more precise. Feather calamus/barbs have shown to be efficient in yielding mtDNA which in turn, provides species identification from scanty and degraded samples. This approach is especially useful in cases of forensic wildlife investigation where the feather undergoes other tests and the integrity of the object must be preserved. Research has also revealed that it is possible to obtain mtDNA and sequence from feather calamus, this is non-destructive and retains the morphological features of the samples. Also, improvements in DNA barcoding, where a specific mitochondrial gene known as COI is applied, has made it easier to distinguish between closely related species and even identify cases of hybridization [7]. This methodology proves useful in conservation biology, archaeology, and forensic science where species identification is vital.

4. CONCLUSION

The study shows that DNA barcoding approach is suitable for molecular analysis of the Indian peafowl by using shed feather samples through non-invasive technique. This method does not only help to identify the species correctly but also can be considered as an effective tool in monitoring the wildlife, assessment of the level of biodiversity, and conservation activities. The success of the DNA barcoding in this study reveals the ability of this technique to improve species identification in birds and thus will contribute immensely to the field of molecular ecology.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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