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Original Article

A Non-Invasive Method of Molecular Authentication of 'Shed Feathers' for the Forensically Informative Nucleotide Sequences (FINS) Analysis

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Abstract

Species identification through visual examination and feather morphometrics is a challenging task for law enforcement agencies in enforcing the Wildlife Protection Act (WPA). In the present study, DNA was extracted by a non-invasive method, i.e., from scattered shed feathers of *Antigone antigone antigone*, the Indian Sarus Crane, in the paddy fields collected during the field survey. Three mitochondrial genes (COI, Cytochrome b and 16s rRNA) were amplified using universal primers for species identification. The homologous sequences were retrieved using NCBI-GenBank for each generated sequence of the three genes. Neighbour-Joining and Maximum Parsimony trees in FINS analysis identified the species from the individual feather with strong bootstrap support. The study highlighted the importance of shed feathers in identifying the species and their applicability in wildlife forensic cases using the FINS approach.

Keywords: *16s rRNA; COI; Cytochrome B; FINS; Phylogenetic Tree; Wildlife Forensics*

Introduction

Molecular phylogenetics, or the study of the evolution of genetic sequences, employs a phylogenetic tree based on similarities and changes in their genetic and/or physical traits. It provides the evolutionary linkages between various species and gene markers. A combination of both molecular and statistical methods is applied in the phylogenetic analysis to establish the evolutionary relationships between genes and species. Forensically Important Nucleotide Sequences (FINS) are tools for identifying organisms based on their genetic variations. The concept of FINS was proposed to identify the origin of animal food products and has since been extensively applied in forensic investigations (Bartlett & Davidson, 1992). DNA sequencing can retrieve maximum molecular information from a particular DNA region. Polymorphism of the resultant nucleotide sequences provides information to distinguish closely related species from distantly related species and also between genuine food products, meat, medicinal materials and adulterants. Phylogenetic studies of unknown samples together with reference species is the principle behind FINS. Sequence alignment is accomplished using phylogenetic analysis to investigate informative homologous regions for species identification and analysis. The evolutionary history of the taxa is reflected in the creation of phylogenetic trees using techniques like maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis. Tools such as Multalin, MEGA, BioEdit and Clustal W facilitate multiple sequence alignments for building phylogenetic trees.

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Despite reports of population declines of Sarus Cranes and the importance of genetic studies to biological conservation, limited studies have been attempted on the genetic variation among the population of *A. antigone* across their entire distribution using dead-shed feathers. The study also attempts to generate relevant data and genetic uniqueness at the molecular level, along with the phylogenetic structure of the genes in this species. An attempt to assess the genetic diversity of various genes from the mitochondrial DNA (mtDNA), namely, Cytochrome oxidase I (COI), Cytochrome b (Cyt b) and the 16s rRNA genes from the shed feather pools from Etawah and Mainpuri districts of Uttar Pradesh, along with reference to the published sequences from Genbank for the same genes. It focuses on establishing a 'non-invasive' phylogenetic identification system for avian species and assessing the levels of genetic diversity and differentiation among them.

Material and Methods

Shed feather samples were collected from the ground, near the nests and confirmed territories of breeding pairs of *A. antigone* from the Sarsai Nawar Wetland (26.96223ºN, 79.23415ºE), Uttar Pradesh. Since the sex and the developmental stage of the individual bird could not be ascertained by only looking at the feather samples, all the collected feathers from a nest were pooled as a single sample for analysis. Each sample was stored in a labelled dry plastic bag and brought to the lab at ambient temperature for later use. The calamus of good-quality feathers was used for DNA extraction. Genomic DNA was extracted using the Nucleospin Tissue XS Extraction Kit (Macherey-Nagel GmbH & Co., Germany) as per the instructions in the manufacturer's protocol (Dubey & Prakash 2025). The quality and quantity of isolated DNA were analysed following Green and Sambrook (2012).

COI, Cytb and 16s rRNA gene sequences from the extracted genomic DNA were amplified by polymerase chain reaction (PCR) using primers given in Table 1. Genomic DNA amplification was carried out in reaction mixtures of 50 µl. Each reaction mixture contained 10 mM Tris-HCl (pH 8.5), 2µ Taq polymerase, 200 µ dNTPs mix, 1.5 mM MgCl2, and 50 mM KCl, 1 µl each of forward and reverse primer, with 60 ng of template DNA. PCR cycle conditions are given in Table 2. PCR products were electrophoresed in 1.5% agarose gel and DNA bands were visualized under a UV Trans-illuminator using a 100 bp ladder (Takara Bio). All the amplified fragments were sequenced with the forward primer by the dideoxy chain termination method (Sanger's Sequencing Method) (Sanger *et al.*, 1977).

Table 1: *Primer Set used for Amplification of Different Genes of Interest*

Sl.no	Gene of Interest	Primer	Sequence	Reference
1	16S rRNA	16SrRNA-Forward 16S rRNA-Reverse	GCCTGTTTACCAAAAACATCAC CTCCATAGGGTCTTCTCGTCTT	Mitchell *et al*., 1993
2	Cytochr-ome B	mcb398 mcb869	TACCATGAGGACAAATATCATTCTG CCTCCTAGTTTGTTAGGGATTGATCG	Verma and Singh, 2003
3	COI	CF CR	GCACAGGATGGACAGTTTAC ATAGCATAGGGGGGTCTCAT	Hebert & Gregory, 2005

Table 2: *PCR Cycle Conditions*

94°C	94°C	55°C	72°C	72°C
2 min	30sec	45sec	1min	10 min
	35 cycles			

The forward and reverse sequences were subjected to BLAST analysis to cross-check the sequences for the three genes. The sequences were analysed and edited using Chromas and a consensus sequence for both genes was obtained with the help of CLC Sequence viewer. The obtained consensus sequences were rerun in BLAST, and the first ten sequences showing the highest similarity with the query sequence were selected. Multiple Sequence Alignment was done using the multalin software, which is an E-software for sequence alignment. The sequence files were transported to MEGA X

(Kumar *et al.,* 2018) for phylogenetic analysis, where multiple sequence alignment was done by CLUSTAL W. Two kinds of Phylogenic trees were prepared using this alignment

I. Neighbour-joining (NJ) with Kimura 2 Parameter, which takes into account the unequal rates of evolution of transition and transversion but assumes an equal distribution of nucleotide composition and

II. Maximum Parsimony (MP) with stepwise addition (1000 replicates) in heuristic search.

All trees were subjected to bootstrap analysis with 1000 replicates to get bootstrap value support. Nucleotide composition and species-specific variable sites were also studied using MEGA X for each of the three genes.

Results

COI gene Analysis. Genetic analysis was done by MEGA X with ten sequences, comparing 623 bp. In relation to the complete mitochondrial genome of *Antigone antigone antigone* out of 623 bp cytochrome oxidase I sequence, there were 599 conserved sites, 12 variable or polymorphic regions and no parsimony and singleton sites (figure 1a). The nucleotide compositions of the entire sequenced region of COI (623 bp) are A 29.3%, T/U 26.4%, C 29.3%, and G 15.1%. Phylogenetic study by using maximum parsimony and neighbour joining algorithms (MEGA X) produced congruent trees (figure 1b) where the target feather pool forms a subclade with accession number 020581.1 with a high bootstrap support of 80 to 86% in both algorithms.

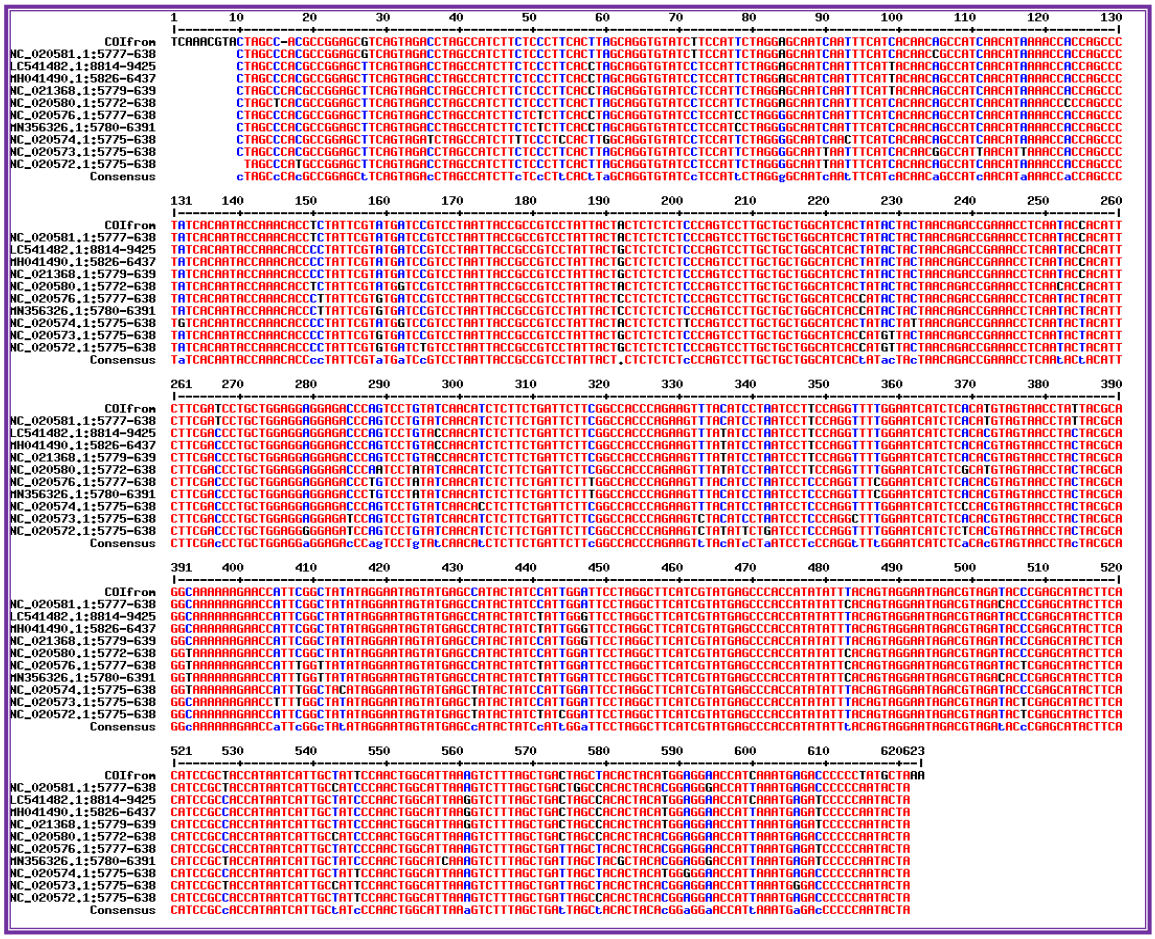


Figure 1a. Multiple Sequence Alignment of COI gene sequence of the shed Feather pool of Sarus Crane collected from the Sarsai Nawar region with reference sequences retrieved from NCBI GenBank where red showed conserved sites, blue showed variable sites and black denoted neutral sites

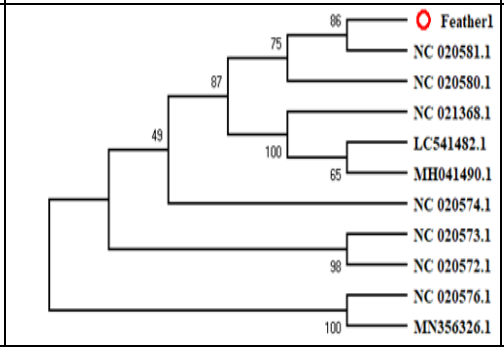
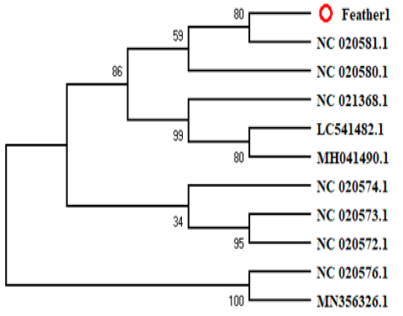
Phylogenetic Trees	***Maximum Parsimony Method***	***Neighbour Joining Method***
COI Gene		

Figure 2a. Phylogenetic trees of the *COI* gene sequences using a) Maximum Parsimony method and b) Neighbour Joining method generated using MEGA X.

Cytochrome b Gene Analysis. Genetic analysis was done by MEGA X with ten sequences, comparing 142 bp. In relation to the complete mitochondrial genome of *Antigone antigone antigone* (NC020581) out of 142 bp cytochrome b sequence, there were 22 conserved sites, 115 variable or polymorphic regions and no parsimony and singleton sites (figure 2a). The nucleotide compositions of the entire sequenced region of COI (623 bp) are A 28.7%, T/U 25.2%, C 34.8%, and G 11.2%. Phylogenetic study by using maximum parsimony and neighbour joining algorithms (MEGA X) produced congruent trees where feather 1 formed a subclade with accession number U11061.1. The maximum parsimony method showed a bootstrap support of 44% for this branching while the neighbour joining method showed a value of 80% bootstrap support for the same subclade (figure 2b).

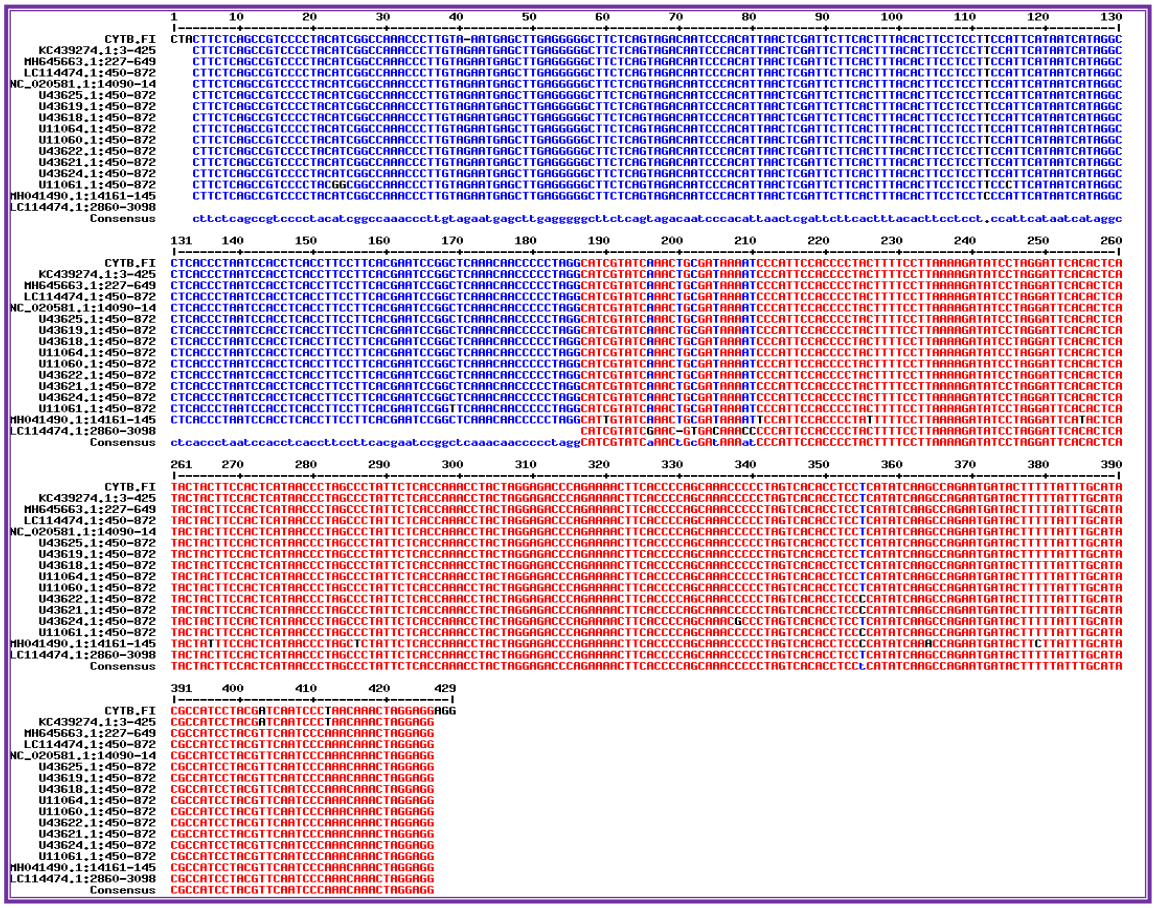


Figure 1b. Multiple Sequence Alignment Cytochrome b gene sequence of the shed Feather pool of Sarus Crane collected from the Sarsai Nawar region with reference sequences retrieved from NCBI GenBank where red showed conserved sites, blue showed variable sites and black denoted neutral sites

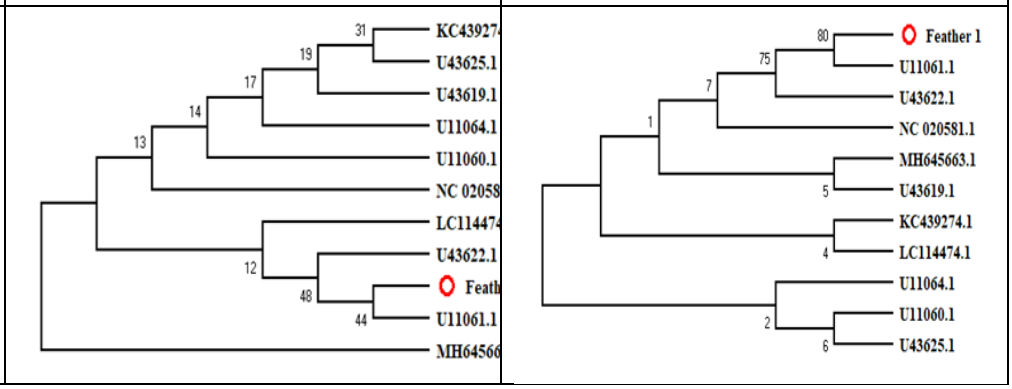
Phylogenetic Trees	***Maximum Parsimony Method***	***Neighbour Joining Method***
Cytochrome b Gene		

Figure 2a. Phylogenetic trees of the *Cytochrome b* gene sequences using a) Maximum Parsimony method and b) Neighbour Joining method generated using MEGA X.

16s rRNA Gene Analysis. Genetic analysis was done by MEGA X with ten sequences, comparing 611 bp. In relation to the complete mitochondrial genome of *Antigone antigone antigone* (NC020581) out of 611 bp 16s ribosomal RNA sequence, there were 231 conserved sites, 341 variable or polymorphic regions and no parsimony and singleton sites (Figure 3a). The nucleotide compositions of the entire sequenced region of COI (611 bp) are A 24.4%, T/U 32.9%, C 20.6 %, and G 22.1 %. Phylogenetic study by using maximum parsimony and neighbour joining algorithms (MEGA X) produced incongruent results with low to moderate bootstrap supports (20 to 50%) with the branching (Figure 3b).

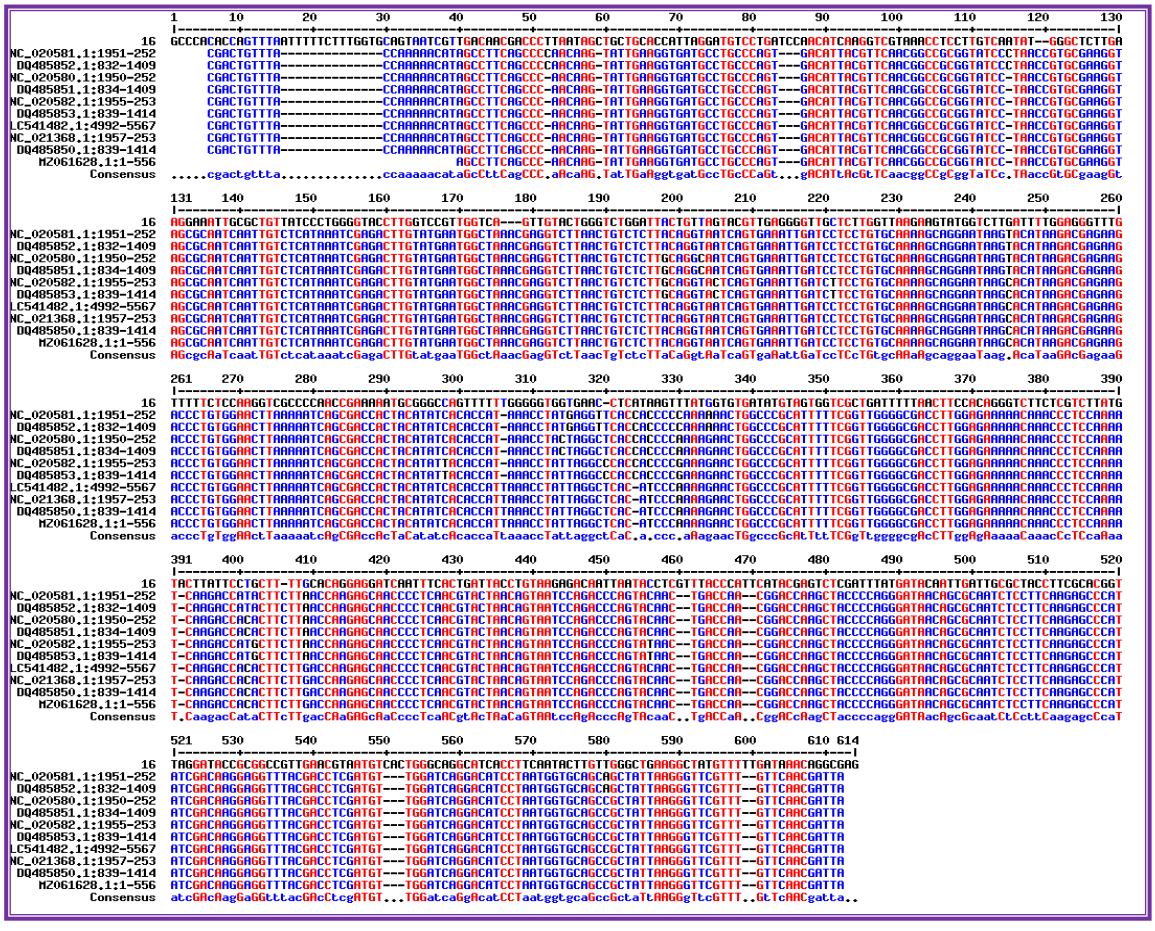


Figure 1c. Multiple Sequence Alignment 16s rRNA gene sequence of the shed Feather pool of Sarus Crane collected from the Sarsai Nawar region with reference sequences retrieved from NCBI GenBank where red showed conserved sites, blue showed variable sites and black denoted neutral sites

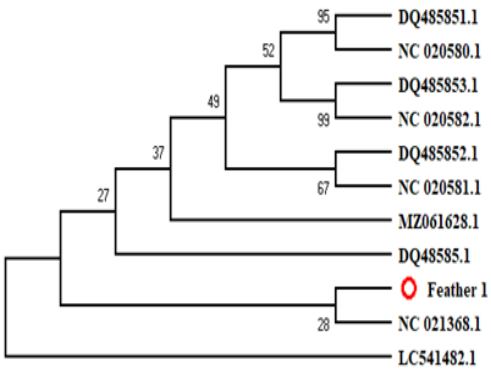
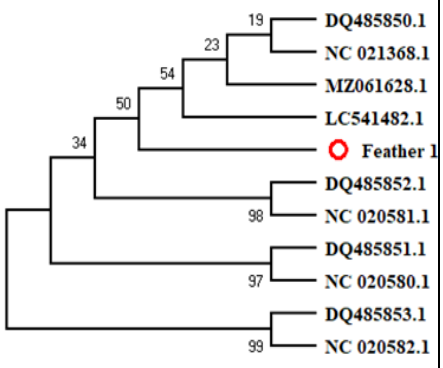
Phylogenetic Trees	***Maximum Parsimony Method***	***Neighbour Joining Method***
16s rRNA Gene		

Figure 2c. Phylogenetic trees of the*16s rRNA* gene sequences using a) Maximum Parsimony method and b) Neighbour Joining method generated using MEGA X.

Discussion

The results of the study discovered the utility of shed feathers to amplify the mitochondrial genes and the potential of FINS technology in identifying the species. Many mitochondrial genes have been used to identify species across taxa, including regulatory areas, 12S rRNA, 16S rRNA, Cytochrome Oxidase I (COI), Cytochrome b (Cyt b) and other indicators (Hajibabaei *et al*., 2006). One of these commonly used approaches in wildlife forensics (Sahajpal & Goyal, 2010; Li *et al*. 2011, Thakur *et al*. 2013, Rajpoot *et al*. 2017; Singh *et al*. 2020), referred to as Forensically Informative Nucleotide Sequencing (FINS), and is also applied for species diagnosis based on particularly informative nucleotide sequences (Bartlett & Davidson, 1992). Over time, replacement in canned goods, marine life and seafood has been identified using FINS in several mitochondrial genome genes (Chapela *et al*., 2002). A number of species, such as lizards and Indian civets, have been identified using FINS (Guha & Kashyap, 2006). Enough information is available at these variable locations to enable interfamilial species distinction.

In FINS analysis, identification of an unknown sample can be performed when the sequence of a gene from an unknown sample is introduced in the estimation of genetic distance among a set of reference sequences and a dendogram (tree) is drawn based on the distance matrix. In this method, the unknown sample will cluster more closely with the same species (Bartlett & Davidson, 1992). The degree of similarity between two sequences is indicated by their genetic distances. If the unknown sequence is included in the calculation of the genetic distance between groups of reference sequences, it is possible to identify any unknown sample. When compared to the species groups to which it belongs, the unknown sequence will have the lowest distance value compared to any other sequence groups. The first two gene sequences in this investigation had congruent trees, unlike the 16s rRNA, due to a less conserved form of this gene found in eukaryotes as compared to bacteria, that has a different evolutionary route compared to the final genetic distances from the reference sequences in the Indian Sarus Crane feather pool sample used here.

The present study uses both DNA distance-based methods (neighbour-joining method), which are preferred for FINS as it simply works on genetic distance from multiple sequence alignment and also phylogeny-based methods (maximum parsimony method) that invoke evolutionary indications to give an idea of any convergent evolution to build a phylogenetic tree based on the COI, Cytochrome b and16s rRNA sequence of the Indian Sarus Crane feather pool. This approach will be particularly useful for wildlife forensics to identify the species from feathers; otherwise morphometric determination of the species using feathers or meat can be inaccurate and needs enormous field expertise. This study also indicates that the Sarus Crane species from India can be identified and differentiated by using molecular tools and techniques using only shed feathers.

However, the homologous sequences from NCBI/GenBank should be retrieved after proper examination as this may often give erroneous results when the sequence of the species in question is not available in the NCBI database and subsequently, there is a high possibility that the sequence of the next closely related species may be retrieved. It is therefore recommended to generate sequences for more than one gene for the sample and then find homologous sequences for each gene on NCBI/GenBank or any other database. This helps minimize the possibility of retrieving wrong sequences and subsequently decreases the chances of misidentifying the samples in question.

Conclusion

Forensically Instrumental Nucleotide Sequences (FINS) Technique was conducted using three gene sequences obtained from the shed feathers of the Sarus Crane using maximum parsimony and neighbour-joining methods. Congruent trees were obtained only for the COI and Cytochrome B genes, but discrepancies were seen in the case of the 16s rRNA gene sequence.

Conflict of Interest

There is no conflict of interest among the authors.

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