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Identification of Galliformes through Forensically Informative Nucleotide Sequencing (FINS) and its Implication in Wildlife Forensics

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Abstract

Galliformes are hunted for the demand of their attractive feathers and to supply a cheap animal food for the rural communities. In such cases, species identification through visual inspection of the meat or based on feather morphometrics is a challenging task for the law enforcement agencies to enforce the Wildlife Protection Act (WPA). Here, we extracted DNA from the individual feathers of unknown species encountered during field surveys and two mitochondrial genes (*12S rRNA* and *Cytochrome b*) were amplified using universal primers for species identification. Most homologous sequences were retrieved using NCBI-BLAST for each generated sequence of both the genes. Neighbor-Joining trees based on Kimura 2 parameter distance matrices in FINS analysis identified the species from the individual feather with strong bootstrap support. Nine species specific polymorphic sites were found in the partial sequence of *Cytochrome b* gene that differentiated *Pavo cristatus* to *Pavo muticus imperator*. Our study highlighted the importance of feathers in identifying the species and their applicability in wildlife offence cases using FINS approach.

Keywords: Galliformes; FINS; *12S rRNA; Cytochrome b;* Species identification

Introduction

Galliformes that commonly referred as a 'gallinaceous birds' are popular for their attractive bright plumage, shy and elusive behavior. They comprise of 70 genera and 284 species worldwide [1]. In India, 45 species of galliformes have been reported which includes one megapode, 27 partridges, quails, francolins and snow cocks and 17 pheasants. Of these, seven species are endemic to India and the global status of 12 species is categorized as 'threatened'. This is largely due to habitat loss, degradation and poaching [2]. In India, illegal poaching of birds is silently on in many protected areas because of the demand of their colorful feathers and cheap source of animal protein for the rural communities that live nearby the Protected Areas. Hunters kill birds for meat and trade their feathers resulting to leave the young orphan chicks to sustain alone in the forest that cause a significant decline in galliformes population across their distribution range in India. Lack of stringent measures to put a check on their poaching is making the situation worst. Therefore, the problem becomes amplified for the law enforcement agencies who are involved in determining the species of the seized material to enforce the wildlife protection act. In past, species identification is generally carried out by immunological methods [3] but with the advancement of the new technologies, nowadays, both nuclear and mitochondrial genes have been targeted for species identification. Highly conserved species specific mitochondrial genes *viz. 12S rRNA*, *Cytochrome b* and *16S rRNA* are amplified using universal primers for identifying species from the seized biological material [4-10]. This approach is popularized as 'Forensically Informative Nucleotide Sequencing (FINS)' [11,12]. In the present study, we tested individual feathers for amplification of *12S rRNA* and *Cytochrome b* genes and their applicability in species identification using FINS approach.

Materials and Methods

DNA extraction and PCR amplification

Fallen feathers from 24 birds of unknown species and pulled feathers from a dead Blood Pheasant (*Ithaginis cruentus*), were collected during field surveys (2008-2010) in different Protected Areas of Uttarakhand state. Feathers of a Silver Pheasant (*Lophura nycthemera*) were collected from a captive bird that was kept for display in Bharat Ratna Pt. Govind Ballabh Pant High Altitude Zoo, Nainital, Uttarakhand. DNA was extracted from the individual feather follicle (*ca.* 0.5-1 cm) which remained attached to the calamus of the individual feather using Qiagen DNeasy tissue kit (Qiagen, Germany) following manufacturer's protocol with slight modifications as suggested by us elsewhere [13]. Two mitochondrial markers *viz. 12S rRNA* and *Cytochrome b* genes were amplified using the universal primers [14]. All PCR reactions were performed on Applied Biosystems thermal cycler (ABI, 2720) in a reaction volume of 10 µl containing 1X PCR buffer (50 mM KCl, 10 mM tris–HCl), 2.5 mM of $MgCl_2$, 200 µM of each d-NTP, 1.25 µg BSA, 4 pM of each primer and 0.5 U of Taq DNA polymerase (MBI, Fermentas) and approximately 15-20 ng of genomic DNA. The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 mins, followed by 35 cycle of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, primer extension at 72°C for 1.5 min. with a final extension at 72°C for 10 min. After amplification, 4 µl of PCR products were subjected to electrophoresis on 2% agarose gel and visualized over transilluminator to detect the amplification.

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DNA sequencing

The PCR products were cleaned up using Exo-SAP treatment to remove residual oligonucleotides and dNTPs prior to DNA sequencing. Forward primer of the universal primers of *12S rRNA* (12SFwd 5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') and *Cytochrome b* gene (CytbFwd 5'-AAAAAGCTTCCATCCAACATCT-CAGCATGATGAAA-3') were used for setting up the cycle sequencing reaction using the Big dye terminator cycle sequencing kit® v 3.1. The sequencing products were cleaned up to remove any unbound ddNTPs using alcoholic precipitation method and subjected for sequencing to ABI 3130 Genetic Analyzer (Applied Biosystems).

Sequence analysis

Qualities of sequences were determined using Sequence Analysis v 5.2 software (Applied Biosystems) and validated by Sequencher v 4.7 software (www. genecode. com). All good quality sequences were compared with NCBI/GenBank (http://www. ncbi. nlm. nih. gov/) database using BLAST tool and most homologous sequences were retrieved from NCBI database. Multiple sequence alignment (MSA) was performed using CLUSTAL W as implemented in BioEdit v 7.0.9.0 software [15]. The phylogenetic trees were generated based on Kimura 2 parameter distance matrix for both the genes using neighbor joining method for all the aligned sequences in Mega v 5.0 [16]. The complete mitochondrial genome of *G. g. murghi* (GU261709.1) was retrieved from NCBI database and aligned with the sequences generated in the present study to identify the species specific polymorphic sites or SNPs.

Results and Discussion

Out of 24 different feather samples, good quality DNA could be extracted from 10 samples and rest samples probably might have suffered by microbial activity prior to sampling, therefore, they did not yield enough DNA. Seven feather samples showed visible bands and both the mitochondrial genes, *12S rRNA* and *Cytochrome b* were sequenced for three feather samples (Table 1).

Feathers 'B', 'C', 'D' and 'F' were identified using *12S rRNA* gene as *Gallus gallus murghi*, *Francolinus pondicerianus, Pavo cristatus* and *Lophura nycthemera,* respectively with strong bootstrap support in FINS analysis while feather 'A' and 'E' did not amplify for *12S rRNA* gene (Figure 1). Feather 'A', 'B', 'C'. 'D' and 'E' were identified using *Cytochrome b* gene as *Lophophorus impejanus, Gallus gallus murghi*, *Francolinus pondicerianus, Pavo cristatus* and *Pavo muticus imperator,* respectively with strong bootstrap support in FINS analysis while feather 'F' and 'G' did not amplify for *Cytochrome b* gene (Figure 2). Overall, FINS analysis of the mitochondrial *12S rRNA* and *Cytochrome b* gene sequences generated from all the analysed species revealed relatively high inter specific variability when compared to intra specific variability.

Sequences of the eight identified species were submitted to the NCBI/GenBank database (Table 2). Interestingly, *12S rRNA* partial gene sequence of *Ithaginis cruentus* and *Lophura nycthemera* were found to be novel and these sequences were not available on NCBI database prior to our submission. The feathers of these two species were collected from the individuals of known identity.

Feather ID	*12S rRNA*	*Reference Accession no.*	*Cytochrome b*	*Reference Accession no.*	Species identified using FINS
Feather-A	X	---	√	gbAF028796.1	Himalayan monal (*Lophophorus impejanus*)
Feather-B	√	gbDQ885561.1	√	gbGU261709.1	Red Junglefowl (*Gallus gallus murghi*)
Feather-C	√	gbDQ832103.1	√	gbU90648.1	Grey francolin (*Francolinus pondicerianus*)
Feather-D	√	gbAY722396.1	√	gbDQ010649.1	Indian Peafowl (*Pavo cristatus*)
Feather-E	X	---	√	gbDQ010650.1	Green Peafowl (*Pavo muticus imperator*)
Feather-F*	√	gbEU417810.1	X	---	Silver Pheasant (*Lophura nycthemera*)
Feather-G*	√	N/A	X	---	Blood pheasant (*Ithaginis cruentus*)

*feathers collected from the birds of known identity; √ successfully amplified; X not amplified.

Table 1: Identification of forensically informative nucleotide sequencing (FINS) using *12S rRNA* and *Cytochrome b* genes in six galliformes.

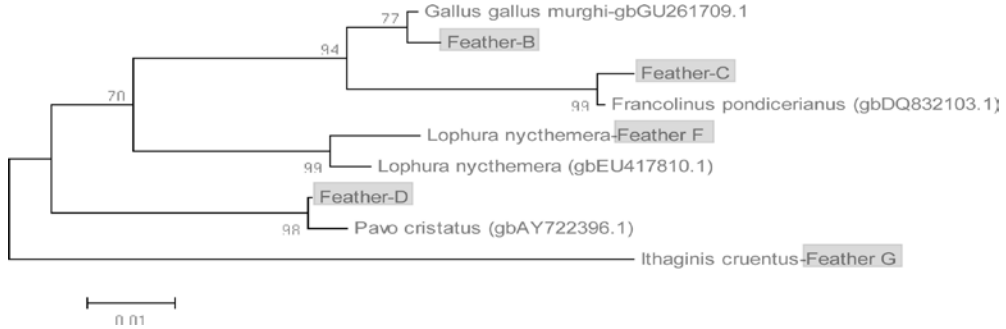

Figure 1: Phylogenetic tree for identification of species from unknown feather samples using 12S rRNA gene by FINS with Kimura-2 parameter distance matrix.

Species specific polymorphic sites in *12S rRNA* gene

In relation to the complete mitochondrial genome of *Gallus gallus murghi* (GU261709.1), there were 28, 27, 32 and 33 nucleotides substitutions in *Ithaginis cruentus, Francolinus pondicerianus, Lophura nycthemera* and *Pavo cristatus,* respectively (Table 3). We found 2 nucleotide additions in *Ithaginis cruentus* and *Francolinus pondicerianus* and single nucleotide addition in *Lophura nycthemera* and *Pavo cristatus*. Seven deletions were found in *Ithaginis cruentus* while there was no deletion in *Lophura nycthemera.*

Species specific polymorphic sites in *Cytochrome b* gene

In relation to complete mitochondrial genome of *Gallus gallus murghi* (GU261709.1), there were 39 and 36 nucleotides substitutions in *Francolinus pondicerianus* and *Pavo muticus imperator*, respectively while 37 nucleotides substitutions were found in *Lophophorus impejanus* and *Pavo cristatus* (Table 4). Single nucleotide addition was found in *Pavo muticus imperator* at position 15330 while no addition of nucleotides was found in *Francolinus pondicerianus, Lophophorus impejanus* and *Pavo cristatus.* No deletion of nucleotide was observed

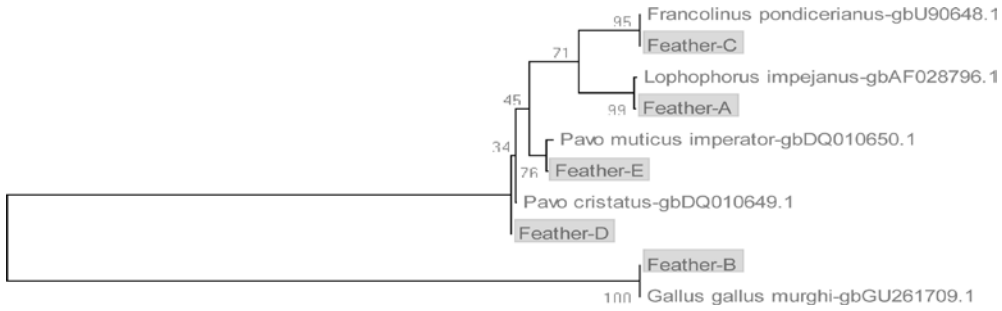

Figure 2: Phylogenetic tree for identification of species from unknown feather samples using *Cytochrome b* gene by FINS with Kimura-2 parameter distance matrix.

Species	***12S rRNA* (Accession no.)**
Francolinus pondicerianus	JQ796700
Pavo cristatus	JQ796703
Lophura nycthemera	JQ796702
Ithaginis cruentus	JQ796701
Species	***Cytochrome b* (Accession no.)**
Lophophorus impejanus	JQ796705
Francolinus pondicerianus	JQ796704
Pavo cristatus	JQ796706
Pavo muticus imperator	JQ796707

Table 2: Submitted NCBI/GeneBank Accession no for the identified galliformess.

***Nt* Position** / **Galliformes**	1778	1779	1781	1783	1784	1785	1786	1787	1790	1791	1792	1793	1794	1795	1803	1852	1883	1898	1903	1919	1920	1939
Gallus gallus murghi	A	C	T	C	C	A	T	C	A	C	A	T	G	T	T	C	T	T	C	G	C	-
Ithaginis cruentus	A	T	C	A	-	-	A	T	C	C	A	C	A	T	T	T	C	T	C	A	C	G
Francolinus pondiceranus	A	C	C	T	C	-	C	C	C	T	A	T	C	T	C	C	T	C	C	G	T	-
Lophura nycthemera	-	-	-	-	-	-	-	-	C	C	A	T	G	C	C	C	T	T	C	A	C	-
Pavo cristatus	G	C	C	T	C	-	A	T	A	C	C	T	A	C	C	T	C	C	T	A	C	-
***Nt* Position** / **Galliformes**	1943	1948	1954	1955	1957	1958	1959	1961	1964	1965	1969	1970	1973	1975	1976	1977	1978	1979	1981	1982	1985	1987
Gallus gallus murghi	C	C	C	T	A	T	G	A	A	A	-	C	T	A	G	C	T	C	A	T	C	C
Ithaginis cruentus	C	C	T	A	T	G	A	A	A	A	A	C	G	A	T	T	A	G	T	C	T	-
Francolinus pondiceranus	T	C	C	C	A	T	G	A	A	A	-	T	T	A	G	C	T	C	A	T	C	C
Lophura nycthemera	C	C	A	A	A	T	G	G	G	C	-	C	T	G	G	C	C	C	A	C	T	T
Pavo cristatus	C	T	C	T	A	T	G	A	A	A	-	C	T	A	G	C	C	C	A	C	T	C

Galliformes \ *Nt* Position	1988	1989	1990	1991	1992	1993	1997	1998	2001	2005	2020	2030	2052	2054	2055	2061	2063	2065	2068	2074	2075	2076
Gallus gallus murghi	C	-	-	T	C	G	A	T	G	G	T	G	A	A	-	C	-	A	A	G	A	C
Ithaginis cruentus	-	-	-	-	-	A	G	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-
Francolinus pondiceranus	C	G	C	C	C	G	A	T	G	G	C	A	A	C	-	T	-	G	A	G	A	T
Lophura nycthemera	A	-	-	C	C	A	A	C	G	G	C	A	A	A	G	C	-	A	A	G	A	C
Pavo cristatus	C	-	-	-	C	A	A	C	G	G	C	A	G	A	-	C	T	A	G	A	G	C

Galliformes \ *Nt* Position	2084	2085	2086	2087	2092	2097	2108	2117	2118	2121	2124	2125	2127	2829	2130	2131	2133	2137	2138			
Gallus gallus murghi	C	G	C	A	G	C	G	A	T	T	A	C	C	C	C	C	T	A	T	C		
Ithaginis cruentus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
Francolinus pondiceranus	C	C	T	G	A	T	A	A	C	T	G	C	C	C	-	-	-	-	-			
Lophura nycthemera	C	G	T	G	G	C	G	G	C	T	A	T	T	T	T	C	T	C	T			
Pavo cristatus	T	G	C	A	G	C	A	G	C	C	A	T	C	C	C	C	-	-	-			

Table 3: Haplotypes and polymorphic sites in galliformes using combined analysis of *12S rRNA* gene with complete mitochondrial genome of *G.g.murghi* (GU261709.1).

Galliformes \ *Nt* Position	15011	15012	15018	15020	15026	15027	15028	15029	15032	15039	15044	15052	15061	15063	15064	15070	15073	15082	15085	15088	15100	15107
Gallus gallus murghi	A	T	G	C	C	A	T	G	C	C	C	A	C	T	G	C	A	A	C	A	C	C
Francolinus pondicerianus	-	-	-	-	-	-	-	-	C	C	C	C	A	C	A	T	A	T	T	C	C	T
Lophophorus impejanus	C	C	A	A	C	A	T	C	C	C	T	C	T	T	A	C	C	C	C	C	T	C
Pavo cristatus	A	C	G	A	T	G	C	C	T	A	T	A	A	T	A	C	C	A	C	A	A	C
Pavo muticus imperator	G	C	G	A	T	G	C	C	T	A	T	A	A	T	A	C	C	A	C	A	A	C

Galliformes \ *Nt* Position	15109	15112	15115	15118	15121	15124	15127	15139	15145	15148	15151	15154	15160	15163	15166	15181	15184	15193	15199	15205	15206	15211
Gallus gallus murghi	C	T	C	G	C	A	A	C	G	T	C	C	C	C	C	C	T	T	C	A	G	A
Francolinus pondicerianus	T	A	C	G	C	A	G	A	A	C	C	T	C	C	C	C	C	T	T	A	G	A
Lophophorus impejanus	C	A	C	A	T	T	A	C	A	T	C	T	C	C	C	C	C	T	C	C	A	C
Pavo cristatus	C	A	T	A	C	A	A	C	A	T	T	T	C	A	T	T	C	C	T	C	G	A
Pavo muticus imperator	C	A	T	A	C	A	A	C	G	T	T	T	T	A	T	C	C	C	C	C	G	A

Galliformes \ *Nt* Position	15217	15227	15229	15230	15232	15235	15238	15247	15257	15259	15271	15274	15280	15283	15286	15289	15292	15298	15301	15304	15310
Gallus gallus murghi	C	C	C	T	C	G	A	C	A	C	A	C	C	C	C	T	G	C	T	T	C	G
Francolinus pondicerianus	T	T	G	C	C	A	A	T	A	C	A	C	A	T	T	C	A	C	T	C	T	A
Lophophorus impejanus	T	C	A	T	T	G	G	C	A	C	A	T	A	T	T	C	A	A	T	C	C	A
Pavo cristatus	C	T	A	T	C	A	A	C	A	C	G	C	A	C	C	C	A	C	C	A	C	A
Pavo muticus imperator	C	T	A	T	C	A	A	C	G	T	A	C	A	C	C	C	A	C	T	A	C	A

Galliformes \ *Nt* Position	15313	15330	15339	15345	15369	15378	15384	15388	15396													
Gallus gallus murghi	C	-	C	T	T	T	C	C	G													
Francolinus pondicerianus	C	-	T	C	C	C	A	T	A													
Lophophorus impejanus	A	-	-	-	-	-	-	-	-													
Pavo cristatus	A	-	-	-	-	-	-	-	-													
Pavo muticus imperator	A	C	-	-	-	-	-	-	-													

(Polymorphic sites between Indian peafowl and green peafowl are highlighted in check box)

Table 4: Haplotypes and polymorphic sites in galliformes using combined analysis of *Cytochrome b* gene with complete mitochondrial genome of *G.g.murghi* (GU261709.1).

in any of the studied galliform species. We found nine polymorphic sites between *Pavo cristatus* and *Pavo muticus imperator* on positions 15011, 15145, 15160, 15181, 15199, 15257, 15259, 15271 and 15330 in *Cytochrome b* gene and these sites can be used to differentiate *Pavo cristatus* to *Pavo muticus imperator*.

Our results showed the applicability of shed feathers to amplify the mitochondrial gene and the potential of FINS technology in identifying the species. However, the homologous sequences from NCBI/GenBank should be retrieved with caution 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 closely related species may be retrieved. We recommend generating sequence for more than one gene for the sample in question and then finding homologous sequences for each gene on NCBI/GenBank database. This way, one can minimize the possibility of retrieving wrong sequences and subsequently decrease the chances of misidentification the species in question. In FINS analysis, identification of unknown sample can be performed when the sequence of a gene from unknown sample is introduced in the estimation of genetic distance among a set of reference sequence and draw a dendogram based on the distance matrix. In this parsimony (FINS) method the unknown sample will cluster more closely with the same species [11]. This approach will be particularly useful for wildlife forensics to identify the species from feathers otherwise morphometric identification of the species using feathers or meat is confusing, inaccurate and needs expertise.

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