RESEARCH ARTICLE

Characterization of 12S rRNA Gene for Species Identification of Common Indian Wild and Domestic Birds

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Abstract

Poaching is a major threat to birds, mammals and reptiles. The Indian peafowl (*Pavo cristatus*), a 'gallinaceous bird', is the national bird of India and belongs to Schedule I of the Indian Wildlife (Protection) Act, 1972. The bird is often killed for its tail feathers and meat. For the effective implementation of law and order, firm evidences are required against the poachers. Additionally, phylogenetic analysis is required to study the biodiversity of avian fauna for better understanding of genetic evolution. Hence, in the present study, characterization of 12S rRNA gene was performed for genomic analysis and phylogentic relationship of Indian peafowl with other free-ranging and domestic birds. A total of 54 Forensically Informative Nucleotide Sequences (FINS) were identified in the present study for identification of species of the panel birds. Indian wild/free-ranging birds and domestic birds were markedly found to be genetically diverse (0.129 ± 0.013) from each other. The phylogenetic analysis was inferred using the Neighbor-joining tree in which different wild bird species were found to be restricted to different clades. Data obtained in the present study using partial fragment of 12S rRNA gene for tissue and faecal samples might be a useful and convenient tool for species identification and can be readily applied to other bird assemblages, making them particularly relevant to a broad range of further avian research.

Key words: Birds, FINS, Forensic, Phylogeny, Wildlife.

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Introduction

Today, India's biodiversity is in jeopardy and many wild species are at the verge of extinction. Poaching is a major threat to birds, mammals and reptiles. In India, poaching of birds has been going on in-and-round forest areas because of the demand for their colorful feathers, and as a cheap or exotic source of animal protein for rural and urban communities. The Indian peafowl (*Pavo cristatus*), a 'gallinaceous bird', is the National bird of India and belongs to Schedule I of the Indian Wildlife (Protection) Act, 1972, is killed for its tail feathers and meat. Differentiation of meat of such protected species is essential in order to address the ambiguity about the origin of the sample. Wildlife DNA forensics is an applied field that has emerged from a synthesis of conservation genetic research and forensic genetic practice to meet the increasing need for investigative tools in wildlife law enforcement. Mitochondrial DNA is also preferred because there are approximately 2 to 10 thousand copies of mtDNA in each mitochondrion and there are hundreds to many thousands of mitochondria per cell, hence, it is possible to obtain a full mtDNA profile from more degraded or deteriorated samples or very small samples (Tobe and Linacre, 2008; Thakur, 2014).

Mitochondrial 12S and 16S rRNA genes have been mostly applied in studies of higher categorical levels such as phyla and family, respectively (Arif *et al*., 2011). These sequences have been useful for inference of moderate to long divergence times (Thakur *et al*., 2013; Kumar *et al*., 2016). Of the rRNA genes, 12S mitochondrial rRNA gene has been widely used to study the phylogenetic relationship among different level of taxa (Verma and Singh, 2003; Rajput *et al*., 2013; Ghosh *et al*., 2019). They have a greater sequence variation than Cytochrome b gene for a species and the process is robust and well validated. The major reason for using mitochondrial DNA is that all maternal descendents will have same mitochondrial sequence, with the exceptions of mutations, all loci will be linked (Clayton *et al.,* 2004). Based on these facts, the present study was undertaken to amplify and sequence the 12S-rRNA gene of wild (Indian Pea-fowl and Blue rock pigeon) and domestic birds (Geese, Emu, Kadaknath bird, Jabalpur colour bird, White leg horn broiler birds) to identify SNP marker within the 12S-rRNA gene for species identification.

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Materials and Methods

Collection of Samples and DNA Isolation

In this study, the authors have examined 12S rRNA gene of Indian wild and free-ranging birds (Indian Pea-fowl and Blue rock pigeon) and domestic birds (Geese, Emu, Kadaknath bird, Jabalpur colour bird, White leg horn broiler birds) for their identification based on SNP typing. From each species, five samples were collected for investigations. Known meat samples of Indian peafowl were collected during the necropsy, which were available at the School of Wildlife Forensic and Health (SWFH), Jabalpur. Mitochondrial DNA was extracted using DNeasy Blood and Tissue extraction kit (Qiagen, Germany). Faecal samples of blue rock pigeon, emu bird, geese, Jabalpur colour poultry bird and Kadaknath poultry bird were collected from different places of Jabalpur and DNA was extracted from the faecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Germany). Meat samples of White Leg Horn broiler bird and Quail were collected from the local market of Jabalpur and Government Quail farm, Bhopal (Madhya Pradesh), respectively. DNA was extracted using above mention kit.

Quality and purity of DNA were checked by submarine agarose gel electrophoresis on 1.0% agarose in 0.5X TBE (pH 8.0) buffer containing Ethidium Bromide (1%) @ 5 µL/100 mL(Sambrook *et al.,* 1989). The wells were charged with 8 µL of DNA preparations mixed with 6X gel loading buffer dye. Electrophoresis was carried out at 70 V for 45 min at room temperature and DNA was visualized under UV transilluminator gel documentation system (Alpha Innotech, JH India Ltd.). Quantity of DNA was estimated by spectrophotometric method using Nanodrop © spectrophotometer (ND-1000). Optical density (OD) at 260 and 280 nm was also taken using buffer AE (QIAGEN, Germany) as blank. Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. The DNA concentration was determined and samples were diluted up to the final concentration of 50 ng/µL with Mili-Q water.

Amplification of the 12s rRNA Gene

The Universal primers of 12S rRNA gene (forward primer, 5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3'; reverse primer, 5'-GAG GGT GAC GGG CGG TGT GT-3') of genomic DNA (Kocher *et al.*, 1989) synthesized by Imperial Life Sciences, India were used to standardize the PCR using 10 pmol of each primer. The PCR condition included an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min and final extension at 72°C for 10 min using Proflex PCR as per Jadav *et al.* (2014). The PCR products were run on 1.5 % agarose gels and visualized under UV light.

Sequence Analysis

Amplified PCR products were subjected for both forward and reverse sequencing (Chromous Biotech Pvt. Ltd., Bengaluru, India). The mitochondrial 12S rRNA sequences obtained from the wild and domestic birds were compared with NCBI/GenBank database using BLAST tool and most homologous sequences were retrieved from NCBI database. The obtained sequences were grouped with respect to the species specific retrieved reference sequence. Multiple sequence alignment (MSA) was performed using CLUSTAL W (Thompson *et al.,* 1994) in MEGA 6.0 (Tamura *et al.,* 2013). Aligned sequences were analyzed for species specific SNP detection. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The evolutionary distances between groups were computed using the Kimura 2-parameter method (Kimura, 1980).

Results and Discussion

PCR Amplification and Sequence Analysis

All samples obtained from the Indian wild and domestic birds were successfully amplified to expected size of 456 bp (Fig. 1). To achieve enough information about degree of intraspecies heterogeneity among the two wild/free-ranging and six domestic avian species, five individuals each of the panel species were subjected to DNA analysis. Availability of mitochondrial rRNA sequences in databases enables instant sequence comparison (Prakash *et al.,* 2000). The sequences generated from the amplified mitochondrial 12S rRNA regions of Indian wild and domestic birds matched with 98-99% homology with their respective reference sequences. Accession numbers of retrieved sequences of two Indian wild and six domestic birds from GenBank are listed in Table 1.

Table 1: Retrieved sequences of exotic wild and domestic birds analyzed in the present study

Species	Reference Accession No.
Peafowl	KF444060
Pigeon	KJ722068
Red Jungle Fowl	KP211423
Kadaknath	KP211425
Quail	AP003195
Domestic Geese	EU932689
Emu	AF338711

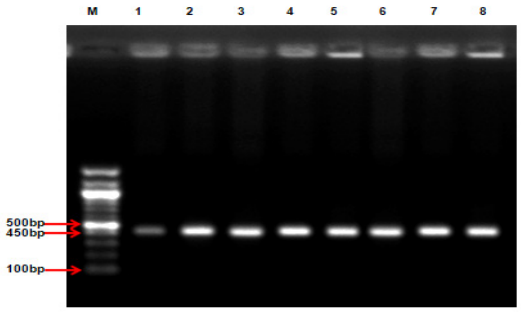

Fig. 1: PCR amplified product (456 bp) of wild and domestic birds. Lane M - 1 kb DNA ladder, Lane 1 - Indian peafowl, Lane 2 - Blue rock pigeon, Lane 3 - Quail, Lane 4 - Geese, Lane 5 - Emu, Lane 6 - Jabalpur Colour Bird, Lane 7 - Kadaknath poultry bird, Lane 8 - White Leg Horn bird.

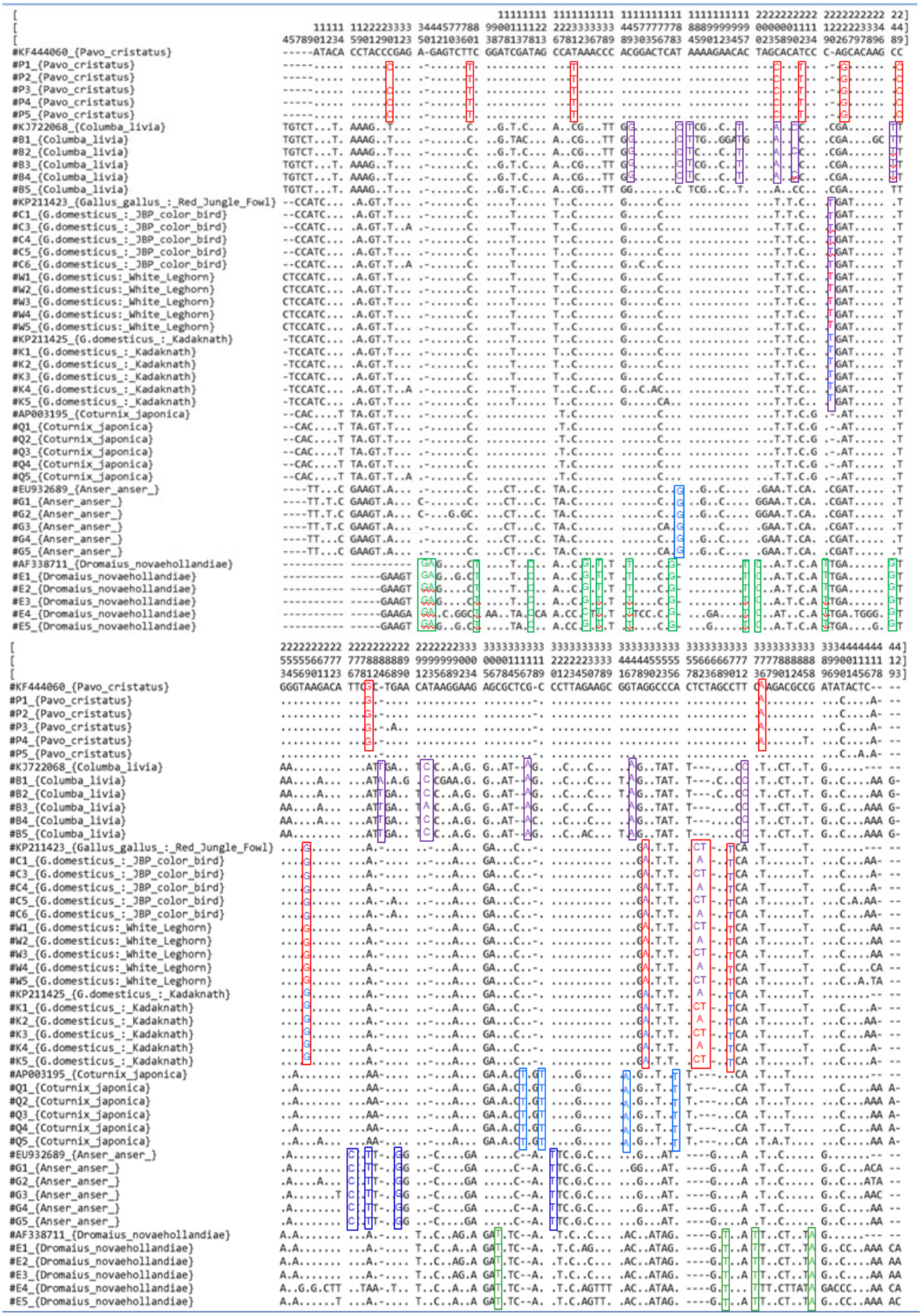

Fig. 2: 12SrRNA sequences from Indian Wild/Free-ranging and Domestic birds were aligned with 7 reference sequences of Indian Wild and Domestic birds to identify 184 polymorphic sites in the examined nucleotide region. A total of 54 unambiguous variations, forensically informative variations (FINS) were observed.

The PCR product (450bp) was sequenced and the sequence obtained was aligned in MEGA 6. The sequence length of different species of birds obtained after sequencing were - 393 bp (Indian peafowl), 400 bp (Blue rock pigeon), 371 bp (Emu), 392 bp (Jabalpur colour bird), 401 bp (White leg horn), 388 bp (Quail), 386 bp (Geese) and 396 bp (Kadaknath). Out of the different sequenced samples the perfectly aligned 343 bp region of mitochondrial 12S rRNA gene fragment obtained from the samples of panel bird species along with the reference sequences of each avian species obtained from GenBank was used for further analysis. Alignments of newly obtained and retrieved sequences for polymorphic and phylogenetic analysis were performed by using MEGA 6.0 software.

Sequences were analyzed to observe unambiguous variations, forensically informative variations (FINS) observed to differentiate wild/free ranging birds from the domestic birds (Fig. 2, Table 2).

In our study, it was observed that the obtained sequences of Indian peafowl, Blue rock pigeon, quail, geese, emu and common poultry breeds showed 09, 13, 04, 05, 16 and 07 unambiguous variations, forensically informative variations (FINS), respectively, whereas Thakur *et al*. (2013) observed 33 nucleotide substitution in *Pavo cristatus* while using only *Gallus gallus murghi* (GU261709) as reference sequence.

Intra-species nucleotide variations were also observed in the newly obtained sequences on alignment as well as on comparison with the retrieved reference sequences. Though the sample size was small, it could be concluded from the study that there were no unambiguous variations and FINS were observed to differentiate individually the three common breeds of poultry from all the sequences examined in the study (Table 3). The average evolutionary distance over sequence pairs within species was also observed as shown in Table 4.

Table 2: List of SNP markers in the four different fragments of the mitochondrial genome of Indian wild and domestic birds as observed in the previous and present studies

Species	Reference Accession No.	Size of the 12S rRNA region	Amplified region	Length of the investigated region (bp)	No. FINS	FINS
Peafowl	KF444060	1215 - 2193	1702 – 2094	393	09	1719 C>T; 1780 T>C; 1819 T>C; 1892 C>A,T; 1964 G>A; 1899 T>C; 1908 G>A; 1951 G>A,T; 2075 A>T.
Pigeon	KJ722068	70 – 1031	54 – 943	400	13	691 G>C; 721 C>T; 723 T>A; 744 C,TA> 754 T>A; 757 C>A; 787 T>C,G; 821 T>A; 829 C>A; 836 C>T; 853 A>G; 882 A>T; 898 C>A,T.
Quail	AP003195	1223 - 2196	1710 – 2096	388	04	2005 T>C; 2011 T>C; 2025 A>T; 2063 T>A
Geese	EU932689	69 – 1056	575 – 959	386	05	744 G>T; 835 C>T; 838 T>G; 848 G>T; 873 T>C; 912 T>C.
Emu	AF338711	71 – 1034	574 – 935	371	14	580 T>G; 585 A>G; 637 T>C; 650 C>T; 666 G>A,C; 687 T>C; 719 G>A; 734 C>T; 744 T>C; 752 T>A; 781 G>C,T; 863 T>C; 893 T>C; 908 T>C; 909 A>G;930 T>C.
Kadaknath, White Leg Horn, Jabalpur colour bird	KP211425 KP211423	1297 – 2272 1297 – 2272 1297 – 2272	1777 – 2172 1773 – 2173 1779 - 2172	396 401 392	07	1983 T>C; 2022 G>A; 2107 G>A; 2112 C>T 2116 T>C; 2117 A>T; 2125 T>C.

Table 3: Intra species variations in the newly obtained Mt-12S rRNA sequences of Indian Wild and Domestic Birds with respect to the retrieved reference sequences

Species	Reference Accession No.	Length of the investigated region (bp)	Amplified region	Size (bp)	Characteristics of the amplified region within species*
Peafowl	KF444060	1215 – 2193	1702 - 2095	393	2095 T>A
Pigeon	KJ722068	70 – 1031	547 - 947	400	944 C>C,A ; 945 C>A; 946 T>A; 947 C>G
Quail	AP003195	1223 – 2196	1710 - 2099	388	2097 T>A; 2098 T>A; 2099 C>A
Domestic Geese	EU932689	69 – 1056	575 - 961	386	960 C>T,T,A; 961 T>A,C
Emu	AF338711	71 – 1034	574 - 945	371	569 C>G; 570 T>A; 571 G>A;572 A>G;573 G>T; 583 G;937 C>A,C; 938 C>A; 939 T>A; 940 C>A,C; 943 A>T; 944 A>C; 945 G>A
Jabalpur Colour Bird	KP211423	1297 – 2272	1779 - 2172	392	2171 C>A, 2172 T>A
WLH Broiler	KP211423	1297 – 2272	1773 - 2173	401	2172 T>A,C,T; 2173 T>A; 2175 A>G; 2176 C>A
Kadaknath	KP211425	1297 – 2272	1777 - 2172	396	2172 T>A

In this study, mitochondrial 12S rRNA gene was used to detect unambiguous nucleotide variations to differentiate amongst Indian peafowl, blue rock pigeon, emu, geese, quail and common poultry birds (Kadaknath, colour bird, white leg horn). It was observed that the partial fragment had the capacity to differentiate the panel bird species through the observed numbers of FINS among the panel avian species. Our results showed the applicability of meat tissue and fecal samples to amplify the mitochondrial gene and the potential of FINS technology in identifying the species. Also the obtained sequences can serve as reference sequences for further analysis as the homologous sequences from NCBI/ GeneBank and if the sequence of the species in query is not available in the NCBI database it subsequently gives rise to a high possibility that the sequence of the closely related species may be retrieved. The present study generated a comprehensive database of DNA sequences from the mitochondrial 12S rRNA genes for panel bird species.

Table 4: Estimates of Average Evolutionary over sequence pairs within species

Species	Distance
Pavo cristatus (pea fowl)	0.004 ± 0.002
Columba livia (Pigeon)	0.021 ± 0.005
Gallus domesticus : Jabalpur colour bird	0.004 ± 0.002
G. domesticus: White Leghorn	0.001 ± 0.001
G. domesticus : Kadaknath	0.005 ± 0.002
Coturnix japonica (Japaneese Quail)	0.003 ± 0.002
Anser anser (Greylag goose)	0.011 ± 0.004
Dromaius novaehollandiae (Emu)	0.061 ± 0.010

Short DNA sequences from the investigated region of the genome provided a number of FINS for identifying different panel species. 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

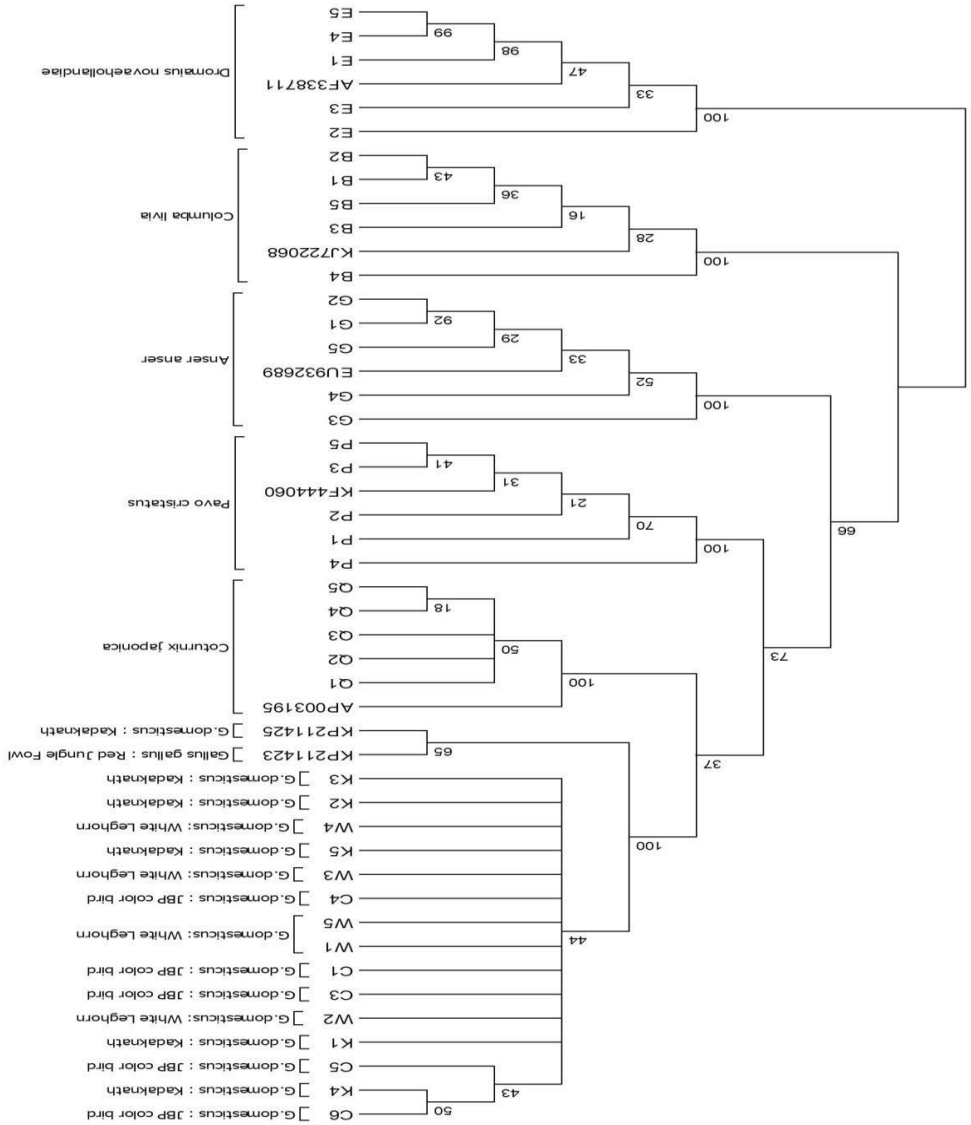

Fig. 3: Neighbor Joining (NJ) Tree on the basis of partial sequence of mitochondrial 12S rRNA gene. Different wild and domestic bird species were restricted to different clades whereas WLH, Jabalpur colour bird and Kadaknath formed a mixed cluster

Table 5: Estimates of Net Evolutionary Divergence between species

Species of bird	Peafowl	Pigeon	Jabalpur Colour Bird	White Leghorn	Kadaknath	Quail	Geese	Emu
Peafowl		± 0.031	± 0.019	± 0.019	± 0.019	± 0.019	± 0.025	± 0.039
Pigeon	0.198		± 0.024	± 0.024	± 0.025	± 0.029	± 0.028	± 0.033
Jabalpur Colour Bird	0.093	0.151		± 0.001	± 0.002	± 0.015	± 0.023	± 0.031
White Leghorn	0.091	0.152	0.003		± 0.001	±0.014	± 0.023	± 0.031
Kadaknath	0.093	0.154	0.004	0.003		± 0.015	± 0.023	± 0.031
Quail	0.096	0.183	0.064	0.062	0.064		± 0.025	± 0.033
Geese	0.156	0.184	0.136	0.134	0.135	0.155		± 0.038
Emu	0.254	0.239	0.206	0.204	0.206	0.216	0.269	

genetic distance among a set of reference sequence and by drawing a dendogram based on the distance matrix as in this parsimony (FINS) method wherein the unknown sample will cluster more closely with the same or near species (Barlett and Davidson, 1992).

Phylogenetic Analysis

The phylogenetic relationship between Indian wild and domestic birds is shown in Figure 3. Evolutionary analyses were conducted in MEGA 6.0 software. The Neighbor-Joining tree demonstrated that each species was restricted to its own cluster. All the breeds of poultry formed one clade. The genetic diversity indices between Indian wild and domestic birds are represented in Table 5.

According to Herbert *et al.* (2004) the neighbor-joining (NJ) tree shows shallow intraspecific and deep interspecific divergence. In the present study, the neighbor-joining (NJ) tree showed shallow intraspecific variations between closely related individuals of the same species like those noted in common local poultry breeds like Jabalpur colour bird, Kadaknath and White Leg Horn whereas deep interspecific divergence was noted in Emu (*Dromaius novaehollandiae*). This was in accordance with the broader classification of aves wherein the class aves are further divided into two subdivisions: the Palaeognathae, which includes the flightless ratites (such as the ostriches, emu etc.) and the weak-flying tinamous, and the extremely diverse Neognathae, containing all other birds (Livezey and Zusi, 2007).

DNA based species identification is on the assumption that individuals from a same species carry specific DNA (or protein) sequences that are different from those found in individuals from other species (Pereira, 2000). In birds, phylogenetic affinities are better understood than in any other large group of organisms. These characteristics make birds an ideal group to analyze the effectiveness of a standardized genetic method for species identification.

In the present study, collectively, these results highlighted the importance of characterizing intraspecific variation by using FINS and phylogenetic analysis for species identification. As the number of studies that utilize different tissue samples continues to rise, genetic species identification will become increasingly important for avian research. The mtDNA fragment employed in the present study was previously identified as an appropriate tool for genetic species identification in birds (Hebert *et al*., 2004), and our results enhanced that claim. As more and more sequences are deposited in public databases (GenBank) and molecular assays for species identification become easier to develop, we envision that molecular techniques will supplement other means of species identification using different tissues in situations where positive identification by visual means is cryptic (Rudnick *et al.,* 2007).

Conclusion

In the present study, a total of 54 unambiguous variations, forensically informative variations (FINS) were observed with respective to the retrieved 7 reference sequences of Indian peafowl, Blue rock pigeon, quail, domestic geese, emu and common poultry birds. Additionally, Indian wild/ free-ranging and domestic birds were found to genetically diverse (0.129 ± 0.013) from each other. The present approach will be particularly useful for wildlife forensics to identify the species and aid their conservation as the purpose of forensic analysis is to provide information or evaluate hypotheses concerning the evidence available. We suppose that our strategy of using partial fragment of 12S rRNA gene for tissue and faecal samples might be a useful and convenient tool for species identification and can be readily applied to other bird assemblages, making them particularly relevant to a broad range of further avian research.

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