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Short Communication

Molecular Identification of Predatory Birds Through Analysis of Mitochondrial *ND2* Gene

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Authors' Contribution
SF gave the idea and developed the whole methodology. RR collected samples and implemented the methodology. MW, ARA and AAA interpreted the results. SS and MT prepared the first draft. MM performed the bioinformatic analysis.

Key words
Coilia nasus, SSR markers, Transcriptome

ABSTRACT

Pakistan is bestowed with variety of habitats and climatic conditions which leads to diverse range of avian species. Among avifauna of Pakistan, Birds of prey or raptors are well-known because of their beauty and speed of flight. Falcons, hawks, kites, eagles, and vulture are common birds of prey. They are geographically widespread and common among the vertebrates. Being predatory birds, they are found on the top of food chain. Unfortunately, birds of prey are facing serious threats such as loss of habitat, pollution, poaching and injuries. In order to maintain ecological balance and food chains, this is very important to make strategies for conservation of these predatory birds. However, there is still uncertainty in their taxonomy because these birds are not studied well at genetic level. Morphological identification includes size, color, and body weight etc. which are crude and does not lead to accurate identification at species level. In order to overcome such gaps, the aim of this study was the identification of two broad families of raptors; *Accipitridae* and *Falconidae*, at molecular level using mitochondrial *ND2* gene. The partial sequence of *ND2* gene was submitted to GenBank. The novel SNPs were investigated which serves as marker for identification of Pakistani raptorial species. Two sub species of falcons are also characterized at genetic level for the first time. The study represented the first report on genetic data of raptorial species of the Pakistan. This strategy can be used to identify other species of birds of prey to get diverse genetic data which will be helpful for the conservation planning of these birds. Developed genetic markers of identification will be used for forensic purposes and also play a significant role in maintenance of ecosystems.

Predatory birds had played important role in ancient civilization associated with the art of falconry. They are tertiary consumer in the food chain showing a wide range of interactions at various trophic levels. Thus, play a significant role in ecological balance. In addition, they are biodiversity indicators of other taxa (Barber *et al*., 2015). Accipitridae is a family of small to large birds with strongly hooked bills and variable morphology. Many well-known birds, such as hawks, eagles, kites, harriers and vultures are included in this group (Jiang *et al*., 2015). Falconidae is a family of diurnal birds of prey which are small to medium-sized. It includes caracaras, laughing falcons, forest falcons, falconets, pygmy falcons and kestrels (Soto *et al*., 2017).

Traditionally, phenotypic characters such as body weight, skin color, eye color, necks, feathers etc. have been employed for the identification of birds. Such systems pose serious challenges as are affected by environment. Thus, these systems are not considered effective for species identification (Coghlan *et al*., 2013).

Mitochondrial DNA is very popular for phylogenetic and other molecular studies due to high copy number, compact architecture and small total size (Ben *et al*., 2017). Among molecular markers, mitochondrial cytochrome c oxidase subunit I (*COI*) has been used for identification of Chilean birds (Colihueque *et al*., 2021). In another study, DNA barcodes has been utilized for identification

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of known species of water birds through *COI* analysis (Pandiyan *et al*., 2022). Although *COI* has been used as standard DNA barcode for identification of birds, NADH dehydrogenase subunit 2 (*ND2*) has been employed as more reliable marker for identification of birds at species level (Luttrell *et al*., 2020). Mitochondrial *ND2* gene encodes an enzyme NADH-ubiquinone Oxidoreductase chain 2 and has comparatively slower rate of evolution. Therefore, it is a best approach for molecular panel development for species and subspecies identification (Uddin *et al*., 2015). Complete mitochondrial *ND2* gene of family *Accipitridae* is 1039bp and of family *Falconidae* is 1041bp long as deciphered from NCBI (www.ncbi.nlm.nih.gov). The main objective of this study was molecular characterization and taxonomic identification of Pakistani raptors through analysis of mitochondrial *ND2* gene.

Materials and methods

Four different species of raptors (5 birds each species) were selected randomly namely; *Accipiter badius* (shikra), *Falco peregrinus peregrinator* (black shaheen), *Falco peregrinus babylonicus* (red-naped shaheen) and *Falco peregrinus* (peregrine falcon) from different regions of Punjab Pakistan in collaboration with the Hawking Club of Punjab and the Global Pet Zone Lahore. About 50-200µL of blood was collected from brachial vein of each adult bird. All the collected samples were labeled as ACCFAL-1 to ACCFAL-20 and stored at -20°C in Molecular Biology and Genomic Laboratory, University of Veterinary and Animal Sciences, Lahore. DNA was extracted from the frozen blood by using standard organic extraction method (Sambrook and Russel, 2001). Primers specific to *ND2* gene were designed through online software Primer3 (http://bioinfo.ut.ee/primer3/) using reference sequence KP336714.1 and NC_000878.1 from NCBI (https://www.ncbi.nlm.nih.gov/) for *Accipitridae* and *Falconidae,* respectively. Amplification of ND2 gene was done using Standard PCR at 58°C. PCR reagents were added in following concentrations; Template DNA (15ng/µl) 30ng/µl, 10x *Taq* polymerase buffer 1x, dNTPs (2.5mM) 0.2mM, $MgCl_2$ (25mM) 1.5mM, primers (10µM) each forward and reverse, *Taq* DNA polymerase 1.25U. The total volume of reaction mixture was 25 µl. Specific temperature profile was used; for both set of primers. The resulted PCR product was visualized by gel electrophoresis (2% gel).

Purified amplicons were subjected to sequencing using dye-labeled dideoxy terminator cycle sequencing through ABI prism 3130 XL Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) following standard protocol.

Sequences obtained after sequencing were analyzed using Electropherogram Chromas Software version (V1.45). For homology analysis, sequences were blast against Reference sequence using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Clustal W (https://www.genome.jp/tools-bin/clustalw) was used for multiple alignment and comparative analysis. Phylogenetic relationship among different raptorial species were analyzed by Molecular Evolutionary Genetic Analysis version 11 (MEGA11) (Tamura *et al*., 2021).

Results and discussion

In this study, four randomly selected species of raptors (5 birds from each species) belong to family Accipitridae and Falconidae were analyzed genetically using mitochondrial *ND2* gene. As a result, we got partial nucleotide sequences of mitochondrial *ND2* gene from 4 species include, *A. badius, F. p. peregrinator, F. p. babylonicus and F. peregrinus,* which were submitted to GenBank NCBI with Accession No. OP719766-OP719769, respectively.

Table I. Novel SNPs in Pakistani *A. badius.*

S. No.	Base position	Allele	Pakistani *A. badius*	*A. b. poliopsis*
1	454	C/T	C	T
2	566	G/A	G	A
3	693	C/T	C	T

Table II. Novel SNPs in Pakistani *F. peregrinus.*

S. No.	Base position	Allele	Pakistani *F. peregrinus*	*F. peregrinus*
1	94	T/A	T	A
2	127	G/A	G	A
3	172	T/A	T	A
4	213	A/T	A	T
5	457	T/C	T	C
6	563	G/A	G	A

Results from NCBI BLAST predicted that the Pakistani *A. badius* show maximum identity with reported sequences of *A. b. poliopsis*. Whereas, we have found 3 novel single nucleotide polymorphisms (SNPs) in Pakistani *A. badius* (Table I). In case of members of family Falconidae, Pakistani *F. peregrinus* is found to be related to already reported sequences and thus, shows 6 novel SNPs at certain position (Table II). The other two members of this family, *F. p. peregrinator* and *F. p. babylonicus*, possess much similarity with *F. peregrinus* but their sequences are not identical to any of the reported species. It seems like these two members have not been identified at molecular level before and therefore, their

nucleotide sequences are not found on NCBI. Our findings compelled us to declare these two members as sub-species of *F. peregrinus*. Evolutionary analysis among various species of raptors through MEGA11 (Fig. 1). The results exhibited that *A. badius* is more closely associated with *A. soloensis* and *A. b. poliopsis*. Similarly, *F. p. peregrinator* and *F. p. babylonicus* has made ingroup with *F. peregrinus*. Whereas Pakistani *F. peregrinus* showed relationship with *F. peregrinus voucher*. The results from phylogenetic tree coincide with the morphological classification of these four species of raptors (Fig. 2). However, it is recommended to explore such species at molecular level further in order to avoid ambiguities.

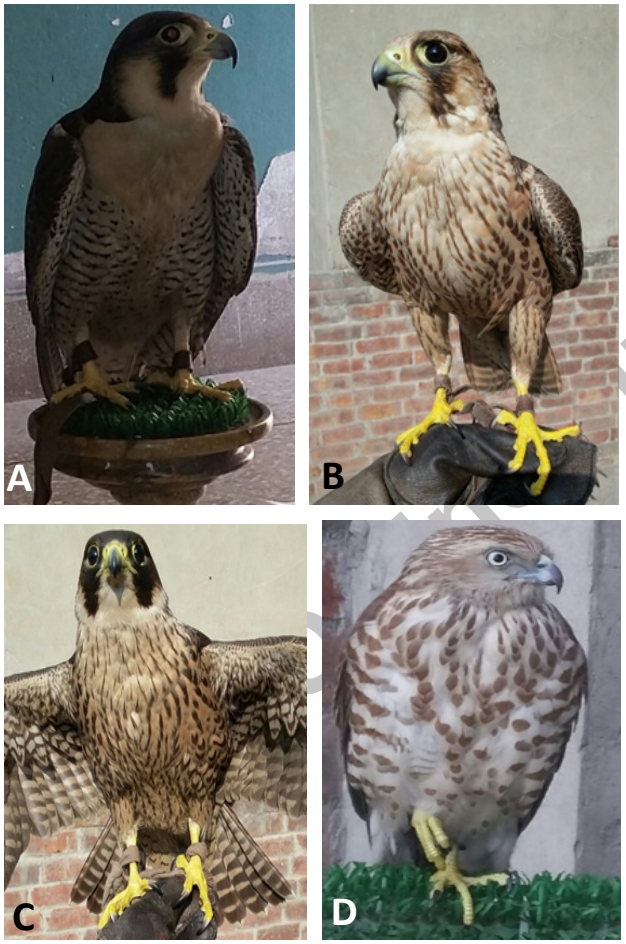

Fig. 1. Predatory birds. A, *F. peregrinus* (Peregrine falcon); B, *F. p. babylonicus* (Red-naped Shaheen); C, *F. p. peregrinator* (Black Shaheen); D, *A. badius* (Shikra).

Similar studies have been conducted by Firyal *et al*. (2014) in Pakistan for the identification of Pakistani domestic pigeon using mitochondrial *Cytb* gene. In another study, molecular approaches have been employed for sex identification in birds (Dubiec and Neubauer, 2006). Although, there is less data available on the raptorial bird species at genetic level, but it was exhibited that there is a dire need for the conservation of certain species of hawks (Bjorklund *et al*., 2015).

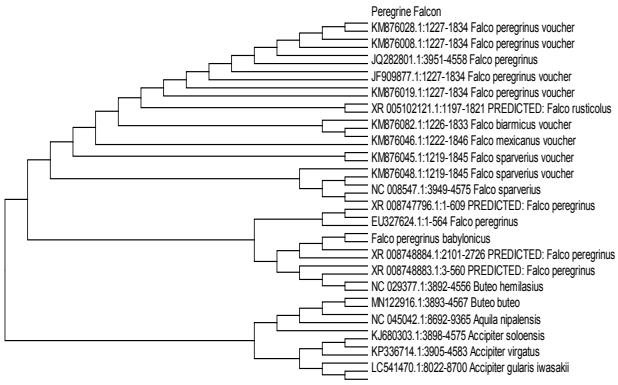

Fig. 2. Phylogenetic tree made through MEGA11.

In the current study, we cannot predict the novel SNPs in the sequences of sub-species of *F. peregrinus* due to lack of records on NCBI. Whereas the novel SNPs of *A. badius* and *F. peregrinus* can serve as markers for the identification of Pakistani raptors. Such kind of study has not been conducted in Pakistan so far.

Conclusion

Partial nucleotide sequences of *ND2* gene of four selected species of raptors was submitted to GenBank. It was found that *A. badius* is closely associated with *A. b. poliopsis* with only three novel SNPs. Whereas, Black Shaheen and Red-Naped Shaheen are sub-species of *F. peregrinus*. Phenotypically different studied Pakistani raptor species are monophyletic. Moreover, Pakistani raptors phylogeny follow the classical ecological divergence pattern.

The developed molecular markers can be used for accurate identification of species as well as for forensic purposes. It can also provide the better conservational activities.

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Ethics statement

All blood samples were carried according to instructions of Animal Ethics Committee of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (DR/ 71 Dated: 08-02-2016).

Statement of conflict of interest

The authors have declared no conflict of interest.

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