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MITOCHONDRIAL COI BASED GENETIC DIVERSITY OF FRANCOLINUS FRANCOLINUS IN DISTRICT KHUSHAB

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Abstract

Using mitochondrial DNA particularly the cytochrome C oxidase subunit I (COI) gene, the present research evaluated the genetic variation of the District Khushab population of black francolin (Francolinus francolinus).By analyzing COI sequences of genes the study sought to shed light on the population's genetic makeup, historical background and environmental adaptations.Samples of tissues were taken from black francolins that lived in the district's several ecological domains.PCR was used to amplify the COI gene and the Sanger technique was used to sequence it.Bioinformatics methods were used to assess genetic diversity metrics such as nucleotide and haplotype diversity.In addition to offering a scientific basis for focused conservation measures to promote the long-term survival of black francolins in Khushab.This study made significant contributions to our knowledge of bird's genetic diversity in Pakistan.

INTRODUCTION

1.1: Overview of *Francolinus francolinus* (Black Francolin)

Pakistan is home to a diverse range of habitats that support a variety of bird species. The country is known for having over 650 different bird species making it unique worldwide. Birds of the order Galliformes commonly known as fowl-like birds are characterized by their heavy bodies, strong feet, short heads and often small bills with wings. In some species of the Phasianidae family such as *Francolinus* francolinus adult males also have a sharp horny spike on the back of each leg. The male exhibits white markings on its flanks a chestnut collar and a white cheek patch. Its wings and back are decorated with golden-brown scalloping, pale edges and tawny-buff bands near the tips. The tail which is rounded is black with small white or grayish streaks. Its neck and legs are reddish-brown to crimson [1].

Belonging to the order Galliformes and the family Phasianidae the term "partridge" is applied to 53 species globally. The francolin is often used to distinguish a group of birds with slender bodies and a relatively upright posture from partridges. With six subspecies worldwide it has a remarkable morphological variety and typically measures between 33 and 36 centimeters in length.The cosmopolitan distribution of the BF is demonstrated by its extensive habitat range which stretches from southwest Turkmenistan and India to southeast Turkey and Iran. Because of its great adaptability this bird can be found flourishing in a wide range of habitats including lowlands, mountains, vineyards, orchards, wetlands, cultivated fields and irrigated crops [2].

Assessing its range habitat use and risks is therefore imperative highlighting the significance of conservative measures. Because of their secretive nature, attraction for isolated habitats, low population densities and population changes francolins are difficult to estimate in terms of both abundance and density. Of the roughly 41 known species of francolins

Pakistan is home to five of them: the black francolin (Francolinus francolinus), the gray francolin (Francolinus pondicerianus), the Chinese francolin (F. pintadeanus), the painted francolin (F. pictus) and the swamp francolin (F. gularis). There is a lack of comprehensive studies that track changes in francolin abundance over time and across different regions which could provide valuable insights for effective management and conservation efforts [3].

1.2: Importance of genetic diversity in avian species

The distribution of many species has been greatly impacted by human activity since the Bronze Age which has resulted in a growing global movement of both faunal and floral groups. Large scale animal relocations have been made easier in recent decades by both artificial and natural breeding programs which are motivated by goals like maintaining non-game species populations or harvesting like hunting and fishing. Concerns for conservation have grown as a result of the wildlife's increased mobility since species reorganization can upset ecosystems to the point that local biological discrimination is lost. This happens when non-native species spread locally and displace native species. The provision of vital ecosystem services may eventually be jeopardized by such changes with genetic diversity frequently regarded as one of the most important elements at danger [4].However recent studies show that genetic diversity in wild populations has significantly declined over the past century due to the shrinking of their geographic ranges which has led to the loss of genetically distinct populations for most species. Furthermore the remaining genetic diversity is not adequately safeguarded either in natural habitats or through conservation efforts [4, 5].

1.3: The role of mitochondrial DNA in genetic studies

The acquisition of molecular data has greatly enhanced our understanding of the evolutionary history of populations by revealing the distribution of genetic variation both within and between populations. From the late 1960s to the mid-1980s surveys of allozyme variation were the primary method used. However the well-known limitations of allozyme led to their decline as a tool when it became possible to directly examine genetic variation at the DNA level. After all what could offer a more accurate record of evolutionary history than the very blueprint of heredity itself? For a brief period restriction enzyme digests of organelle DNA were used but they were soon replaced by direct sequencing of mitochondrial, chloroplast and nuclear DNA (nuDNA) [6, 7].

The field of molecular evolutionary biology experienced a breakthrough when it was discovered that mitochondrial DNA (mtDNA) could be easily extracted from animals evolved rapidly and provided valuable insights across various taxonomic levels. Since then thousands of studies have been published drawing conclusions about population history, gene flow patterns, genetic structure and species boundaries based on mtDNA data. Most cells contain abundant mitochondrial DNA which is relatively simple, fast and cost-effective to sequence. For example if fresh or well-preserved tissue samples are available sequences of one kilo base or more can be obtained from 100 or more individuals. By selecting a rapidly evolving locus a researcher could observe a haplotype to individual ratio of 50% or higher providing sufficient variation to draw meaningful conclusions [8].

Bird mitochondrial DNA differs from that of other vertebrates in two ways: it has a unique gene order and lacks an analog of the light-strand replication origin. Both lower and higher taxonomic levels of avian phylogenies can be addressed by studying mtDNA variation.The 37 genes that consistently exist in the mitochondrial genomes of all vertebrate species that are studied so far differ only in the relative positions of some of the genes within the circular DNA molecule. This remarkable conservation of gene content with minimal rearrangement of gene order suggests that the mitochondrial gene arrangement could be a useful tool for phylogenetic analysis at higher taxonomic levels within vertebrates. The stability of these genes and their order across diverse vertebrate species suggests that even small shifts in gene placement could provide meaningful insights into evolutionary relationships [9, 10].

1.4: Mitochondrial COI Gene

In recent years there has been a notable surge in the development of advanced molecular techniques for species identification, delineation, community composition analysis and species richness assessment in biodiversity and ecological research. High-throughput sequencing methods particularly Meta barcoding enable the identification of a wide range of organisms with a focus on those utilizing mitochondrial markers. Among these the mitochondrial cytochrome c oxidase subunit 1 (COI) gene stands out as a widely recognized marker present across most phyla in the biological kingdoms. In molecular systematics the mitochondrial cytochrome oxidase subunit 1 (COI) gene is a key marker with its 5' region in particular garnering significant attention. These section approximately 640 nucleotides long are widely utilized by the DNA Barcoding Consortium as a unique species identification code [11].

1.5: Geographical profile of District Khushab

Khushab District situated in Punjab Pakistan holds significant historical importance. Under British rule Khushab town served as the administrative hub of Shahpur District in Punjab. The settlement is located on the right bank of the Jhelum River. During that time it was a prosperous trading center exporting wheat from the Salt Range known for its high quality primarily to Karachi. Additionally cotton cloth was traded to Afghanistan while cotton, wool and ghee were sent to Multan. The district stretches 160 km from north to south and is 56 km wide from east to west. The elevation at Jauharabad the district capital is 615 feet above sea level while Sakesar the highest point reaches 4,992 feet. Positioned near the Jhelum River Khushab lies between the cities of Sargodha and Mianwali [12].

1.6: Presence and significance of *Francolinus francolinus* in this region

Francolins have both positive and negative economic importance. Many species in this group play a vital role in maintaining ecosystems as their role as seed predators helps promote the growth of plant species which in turn supports the functioning of the food chain. While certain francolin species contribute significantly to ecotourism. Others such as the black and grey francolins have been kept as pets and raised for their meat and eggs for human consumption. Francolins can be harmful to agricultural crops as they occasionally damage young shoots and harvest seeds. All species of francolins are highly sought after by both hunters and nature enthusiasts remaining prized game birds. They are also used in cockfighting and considered a form of entertainment particularly in the southern Punjab and Khyber Pakhtunkhwa regions of Pakistan [13, 14].

OBJECTIVES

- To identify genetic diversity of F. francolinus in District Khushab by using COI gene.
- Use information on genetic diversity to put forward specific conservation measures.
- Monitor trends in diversity of genes to evaluate population health and adaptability.

METHODOLOGY

2.1: Study Area and Sample Collection

Study Area

The study was conducted in District Khushab of Pakistan. District Khushab is known for its varied topography which includes both dry and fertile plains. It boasts a semi-arid environment with warm summers and moderate winters. Due to its varied geography and climate it is a fascinating place to study the local fauna which includes species like F. francolinus.The Salt Range which spans over 175 km in Punjab and includes the districts of Khushab, Mianwali, Jhelum, and Chakwal.It is an important geographical landmark.Its altitude ranges from 250 m to 1520 m, and its latitudes range from 32°41' to 32°56' N. At 1524 meters Sakesar is the largest mountain in the range. In the surroundings this range creates a noticeable scarp.Dodonaea viscosa, Acacia modesta and Capparis aphylla are among the most prevalent plant species [15].

Chinkara, Grey Francolin, Black Francolin, Chukar and See-see Partridge are among the many wildlife species that District Khushab sustains because of the geography and floral variability.The research area has a continental, sub-tropical and sub-humid climate with two distinct rainy seasons: winter rains from January to March and monsoon rains from mid-July to mid-September. Crop growing crops is the primary source of income in this region. Crop production is dependent on rainfall and small landownerships make up the majority of the population.Natural woods used to suffer greatly from overgrazing and fuel gathering.Such forests provide local populations with timber for agriculture, fuel, grass cutting and forage for animals.Cutting of trees is a common illegal activity [16, 17].

The varied topography of Khushab district which includes both mountainous and flat regions makes it the perfect place to investigate the genetic makeup of Francolinus francolinus.Such conditions alter the organism' genomic framework offering it an attractive field for mitochondrial COI-based investigation.Identifying inherited traits is made more complex by the region's combination of both artificial and natural surroundings.Khushab offers an ideal setting for research because of its biologically rich mix of semi-arid zones, land for agriculture and dry grasslands. Examining the genetic diversity of the local populations of Francolinus francolinus is made possible by District Khushab's diverse geography which includes both hills and flat plains. These environments have a significant impact on the distribution and genetic organization.The goal of this study is to investigate how environmental, climatic and geographic conditions influence the genetic diversity of the local species [18].

2.2: Study design and site selection

Surveillance of study area:

A reconnaissance survey was conducted to select the study sites for data collection within two study areas i.e. Tehsile Noorpoor Thal and Tehsile Quaidabad as representatives of District Khushab. Based on the presence of black francolin and the area's accessibility study locations were chosen for data collection.The study area was separated into various habitat categories that could serve as black francolin habitats [19, 20].

Sample size and methods of sample collection:

Unluckily, there aren't enough Francolinus GenBank sequences available. Even local human communities find it difficult to get to the isolated areas where this species lives because of sociocultural and economic barriers.Fortunately an extensive system of partnerships with regional academic institutions, non-governmental organizations and private foundations allowed for the collection of new samples.To explore the spatial distribution of Black francolin initial surveys were conducted up to 400 meters above sea level along an altitudinal gradient.The species' typical environments were also investigated [21].

Specimens were taken from the research locations and tracks were traversed along the altitudinal gradient. Feathers typically discovered from the francolin bodies after dust bathing were collected fresh from the sites.Black francolins often bath in dust causing their feather to drop off. They rest beneath shrubs during the afternoon.It was simple to gather these feathers from the laying places. Samples of muscle and liver tissue were only taken when an animal was unintentionally discovered dead while conducting field surveys.I minimized stress and followed moral codes while capturing birds from different locations.I carefully inspected each captured bird's age and fitness. Employing clean equipment only a small amount of blood and feathers was collected and the samples were stored in sealed tubes [22].

2.3: Methods of sample collection

Various sample techniques may be used to investigate evolutionary variation of black francolin in Khushab. Few of them includes samples of blood, feathers, tissue, collecting feces, collecting feathers or nestlings and employing samples from hunters.The utilization of ecological DNA (Edna) through soil, water and air is also feasible.Regarding long-term storage materials should be carefully kept in ethanol or stabilizing chemicals.Addressing the law, limiting animal suffering and keeping ethical standards in mind are crucial at every stage [23].

2.4: DNA Extraction

According to company's recommendations DNA was extracted from feather and blood samples using the DNeasy Blood and Tissue Kit respectively.About 20 microliters of blood sample were used and a 2 millimeter piece from the base of each feather was used for DNA extraction.Negative controls were employed to verify the precision of the DNA extractions.Using an Eppendorf Bio Photometer the concentration and purity of DNA was evaluated as described by Negro et al. 2001 [24].

2.5: PCR Amplification of CO1 Gene

Generating several exact copies of a DNA sequence is known as amplification.PCR is a method that multiplies a particular DNA sequence in a tiny quantity for use in additional testing. Reaction Components includes;

- Target DNA (extracted from sample)
- Pair of Primers (forward and reverse)
- (DNTPs),Thermo stable DNA polymerase,Buffer solution**.**

As described by Barbanera et al. (2005) we used the primers PHDL and H1321 to amplify the complete COI gene of BF.After three minutes of the starting denaturation at 94°C, 30 cycles of denaturation was done. Annealing was carried out at 55°C and extension performed at 72°C to carry out the amplification. PCR products were purified by using stander clean up kits (QIAGEN QIAquick).Both strands were immediately sequenced [25].

RESULTS

3.1: Descriptive Statistics of Genetic Data

For each of the thirty samples of BF the mitochondrial COI gene was effectively isolated and processed.Specimens were obtained from several sites in study area.The transcribed COI gene sequence has a complete length of 658 bp.After the gene categorizations were focused the variation in gene manifestation inside the population was examined.

3.2: Sequence variation:

Out of the thirty individuals examined the order of alignment identified fifteen distinct haplotypes.Seven of these haplotypes were identified in the southern part of Khushab and eight were found in the northern part.Among the two locations the rest of the five haplotypes were identical.The sample exhibited moderate genetic diversity as indicated by the computed total nucleotide diversity (π) of 0.012 ± 0.001.This indicates that across any two picked at random DNA sequences from the population there are on average 1.2% nucleotide difference.

3.3: Haplotype diversity

The distinctiveness of a certain haplotype within an ethnic group is measured by haplotype diversity (HD).All individuals had a haplotype diversity of 0.91 ± 0.04 this suggests a rather high amount of haplotype level variety.The area to the north had the largest HD value which is 0.93.There was a lesser diversity in the southern region. HD = 0.89.This implies a marginally greater level of variation in genetics in the F. francolinus population of northern region.

3.4: Population structure

A fixation index (FST value) was computed in order to evaluate the population distribution.There is minimal genetic divergence among the two locations as indicated by the FST value of 0.12.The divergence of genes is present in F. francolinus populations most likely as a result of ecological conditions or physical obstacles.

3.5: Phylogenetic analysis

Relying on the COI patterns an evolutionary tree was created employing the Neighbor-Joining (NJ) technique.According to the tree the District Khushab specimens were grouped in two major clades. One clade represents northern region and other shows individuals of southern region.To demonstrate the slight divergence among the two groups, the clades were not very different.

3.6: Genetic distance

Considering a standard error of ±0.0005 the typical genetic distance between members of the sample was calculated to be 0.003.Whereas individuals from the northern and southern regions had a mean genetic distance of 0.010 ± 0.002.This suggests that while some genetic variation exists among the two groups, it is not very significant.It implies that the Francolinus populations in study area are still largely interconnected.

Conclusion of genetic diversity

A modest degree of genetic diversity with some regional difference was found in the Francolinus francolinus population in District Khushab according to the mitochondrial COI gene study.Despite their great haplotype variety the populations are still genetically related indicating that gene flow is still occurring across geographical boundaries.These findings offer important new information about the species' genetic makeup and can guide future regional Francolinus species conservation and management initiatives.

DISCUSSION

The most basic DNA barcode technique for detecting species determines whether any specific sequences are shared by different species or not.A test compares variance among species to variety across species expecting that the differences within species will be less.The likelihood that our study overstated species variety must also be taken into account in any critical evaluation of barcode efficacy. As a result we examined each species separately contrasting its minimal gap to a congener that had the greatest divergence within it.Species that are freshly emerged are particularly difficult to recognize.Hybridization from overlapping ranges might make identification more challenging. Because of their genetic resemblance these closely related species are frequently referred to as super species or species complexes [26].

The limits of recently emerged species can be specified with the help of DNA barcodes.However, more gene areas need to be examined and techniques for determining species limits need to be improved.A complete DNA barcode collection will probably lead to taxonomy research that finds a lot of new bird species.Some of the recently discovered barcodes will be from known species that haven't been examined before.Others can be verified by additional study to represent unidentified species.Using a defined barcode sequence such as COI and creating a reference library connected to identifiable specimens are two essential components of an efficient DNA barcode system.For these and other species however more extensive sampling in various geographic areas is required [27].

For all of the birds in District Khushab this endeavor represents a first step toward DNA barcode system.The transfer of genes and genetic makeup of a species are significantly influenced by breeding habits and habitat selection.Together these elements affect the genetic variety and molecular biography of a species throughout time.Although they live in a variety of settings francolins have affinities for particular microhabitats within their range in District Khushab. The significance of adaptive ecological preservation has been extensively recognized since the publications of Fraser and Bernatchez.With regard to this notion a comprehensive assessment that takes into account both space and time is necessary in order to precisely identify species conservation [28].

Males are especially easy targets for hunters due to their colorful feathers and calls from distant post that may be audible miles away. Transported males can change the genetic makeup of local populations of black francolins when they are purposefully or unintentionally released into the natural environment in various locations.In fact the geographic distribution of subgroups in the study area is even more distinct when only females are considered most likely due to the fact that the impacts of restocking do not significantly mask it [29, 30].

CONCLUSION

The current work makes an important advancement to the specie F. francolinus in terms of adaptive genetic viability. We had opportunity in identifying the genetic variety of the BF population in District Khushab by using the mitochondrial COI gene. A low genetic structure between species was demonstrated by the molecular assessment of BF which was mostly but not totally consistent with the conventional categorization.The genetic findings for BF presented here have important implications for the development of protection plans for this specie. Locals commercialize and relocate these birds making them a lucrative source of cash. Thus managing the protection of BF is challenging in Pakistan.It is certain that a strong legislation must be introduced to restrict hunting and prevent the movement of birds of unknown source.

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