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RESEARCH ARTICLE

Molecular characterization of Asian Black Francolin (*Francolinus francolinus asiae*) from Western Himalaya based on mitochondrial control region.

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Abstract

Five species of francolins extend their range up to Indian sub-continent *viz*., Asian Black Francolin (*Francolinus francolinus asiae*), Grey Francolin (*Francolinus pondicerianus interpositus*), Chinese Francolin (*F. Pintadeanus*), Painted Francolin (*F. pictus*), Swamp Francolin, (*F. gularis*). So far, as per currently available records only two species, Asian Black Francolin and North Indian Grey francolin of genus *Francolinus* have been reported from Western Himalayan region. Being a game bird, these are under several anthropogenic threats in this region, with very less demographic or molecular data available. The present work aimed to study molecular polymorphism pattern in the Control Region gene (of mitochondrial DNA) so as to obtain some initial data on genetic diversity and probable genetic structure of the population of black francolin in this region. Overall 24 samples were collected from five sites located at different altitudes. We detected 12 polymorphic sites in an average 575bp fragment obtained for the mtDNA Control Region. The average nucleotide frequencies were A=24.54%, T/U=30.03%, C=31.06% and G=14.37% and Transition/Transversion bias (*R*) of 2.79 suggesting this is a recently evolved group or slowly evolving genes. These findings will provide a base line data for future studies on population as well as conservation genetics of francolins from this region.

Introduction:-

The term francolin has been frequently used to separate a group of birds having slimmer body and relatively upright body posture. Francolins are bird that traditionally have been placed in the genus *Francolinus*. *Francolinus* is the largest genus of the family Phasianidae of the order Galliformes (Morony et al., 1975). These are medium-sized, sedentary, old world, partridge/quail-like game-birds adapted to varied but primarily tropical/sub-tropical habitats ranging from dry, lowland grassland to montane forests (Hall, 1963; Johnsgard, 1988; del Hoyo et al., 1994; Madge and McGowan, 2002). They are widely distributed throughout the world occupying different habitats from the high mountains of the Himalayas to dense rain forests of Southeast Asia. Genus *Francolinus* include 41 species of which 5 are restricted to Asia while remaining species are restricted to Africa. Several species have also been introduced to other parts of the world notably Hawaii. Five species which extend their range up to Indian sub-continent are Asian Black Francolin (*Francolinus francolinus asiae*), Grey Francolin (*Francolinus pondicerianus interpositus*), Chinese Francolin (*F. Pintadeanus*), Painted Francolin (*F. pictus*), Swamp Francolin, (*F. gularis*). Painted Francolin (*Francolinus pictus*) is endemic to the Indian Subcontinent (India & Sri Lanka). So far as per the reports from Western Himalaya only two species of genus *Francolinus* have been reported *viz*., Asian Black Francolin (*Francolinus francolinus asiae*) and the North Indian Grey francolin (*Francolinus pondicerianus interpositus*).

Black Francolin is found in India, Pakistan, Nepal, Bangladesh, Sri Lanka, Indonesia, Java, Sumatra and Maldives and Afghanistan and is resident to Kashmir and Northern India (Ali and Repley, 1980). In India black francolin is

distributed over Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Punjab, Uttar Pradesh, Bihar and roughly West Bengal, Madhya Pradesh, Gujarat and Orissa within an altitudinal range from 300 to 2100 asl. Black Francolin occupies diverse habitat types at different altitudinal range and is a non-migratory, traveling short distances (Johnsgard, 1988). It has been reported to occur in the scrub habitats, having plenty of low shrubs and tall grasses (Roberts, 1991) and appear to be closely associated to water and well adapted to cultivated crops, tall enough to offer shelter and open beneath to provide escape routes and easy travel. It has been observed digging/ scratching at the ground (Wijeyamohan et al., 2003), tearing at anthills (Johns, 1980) and pecking cattle dung (Ali and Ripley, 1969) possibly searching for ants and insects, which constitute its food. This species has been regarded as omnivores, consuming seeds of cultivated crops, weeds, green leaves of wild grasses, insects (Ali and Ripley, 1969; Roberts, 1991; Chaudhry and Bhatti, 1992; Wijeyamohan et al., 2003). Global populations of these species were stable and were considered as least concerned (IUCN, 2007; Birdlife, 2007) however recent reports suggest decline due to hunting and habitat loss (The World Conservation Union, 2010).

Differences in altitudinal gradients, topography and landscape characteristics affect the proportion of suitable habitat, influence dispersal patterns. Changing habitat may also influence genetic structure differentiation among populations. Mitochondrial DNA has become a tool of choice for studying molecular evolution and phylogeny of many animal taxa in last few years of researches in systematic (Avise, 1994). The mitochondrial genome of vertebrates is a small, circular molecule of 15-20 kb, with a compact and conserved organization in most, but not all of the studied species (Wolstenholme, 1992). It contains 37 genes: 13 protein-coding genes, 22 transfer RNA genes (tRNAs) and two ribosomal RNA genes (rRNAs) and the control region (D-loop) which controls the initiation of replication and transcription of animal mitochondrial DNA (mtDNA). Because the relatively simple structure, mutation rates are higher than nuclear DNA, thus the mtDNA sequences is used frequently to estimate phylogenetic relationships among animal taxa, population genetics and molecular evolution. In recent years, the sequences of mtDNA have been determined at an increasing pace. Today, 109 avian mitochondrial genomes are now available in GenBank/EMBL/DDBJ. Within Galliformes, about 22 mitochondrial genomes from three major lineages of galliform birds (Megapodiidae, Numididae, and Phasianidae) have been sequenced, and the most recent record is Cabot's Tragopan, *Tragopan caboti*, which was determined by Kan et al., (2010).

During the present study molecular characterization of black francolin was performed, which is observed in a large altitudinal ranges in Western Himalaya in between altitude 300m to 2100m asl occupying different habitat types. Although studies have been performed by several researchers regarding habitat ecology and population biology lesser work has been done at molecular level with meager information available for this species. As far as, the studies on francolin in this region is concerned almost no information is available on mitochondrial DNA based molecular characterization. The present study is aimed to know the genetic structure of black francolin using mitochondrial control region, along a vast distribution ranges across the altitudinal gradient.

Materials and methods:-

Study area:-

The atmospheric temperature is the dominant factor in Himalaya that determines the wide range of ecological conditions, the altitudinal zonation, gradations of ecosystems, distributional patterns and other peculiarities of the biogeography of the Himalaya. This fundamental difference determines the wide difference in the ecology and distribution of animals and plants in the eastern and western ends of the Himalaya. The samples were collected from North-western Himalayan region, located between 28º43'-31º27' N latitudes and 77º34'-81º02' E longitudes.

Study organism:-

Under genus Francolinus only two species has been recorded so far from Uttarakhand, Black francolin and Grey Francolin. Black francolin (*Francolinus francolinus asiae*) is a medium sized Gallinaceous bird having a body length of about ~34 cm and ~227-566 gm in weight with clear sexual dichromatism. Male have ear covered patch on otherwise black face rufous collar, black upper mantle spotted with white and black under part with flanks boldly spotted with white. The female is paler mainly brown. The upper parts are dark brown with pale edged feathers and juvenile resembles females with less marked plumage.

Sample collection :-

Non invasive samples like feathers, egg shells and pellets were collected from the wild for DNA extraction. Tissue samples were collected from the animals found dead during field surveys. Feathers, eggs shells were preserved in air tight zip lock bags containing silica gel and Sodium Chloride. Tissue samples were preserved in absolute ethanol.

A total of 24 samples were collected from different location with different habitat types. The five different sampling sites in this study were located at different altitudinal ranges and significantly isolated from each other.

DNA Extraction:-

DNA from feathers and tissue was extracted using Chelax and QIAgen DNAeasy® Tissue Kit. Following protocols were used for non invasive isolation of DNA using feathers in most of the cases, and liver tissue in some cases where the bird was accidentally found dead while extensive sampling surveys. Feathers base (calamus) approximately 5 mm was cut and placed in a 0.8 ml tube containing 250 µl sterile 5% Chelex® (Bio-Rad). Samples were then incubated at 100°C for 15 min; vortexed twice for 15 sec during incubation period and was allowed to cool at room temperature and then spinned for 30 sec at 14000 rpm. The supernatant was transferred to a fresh, sterile 0.8 ml centrifuge tube and stored at 4°C.

PCR Amplification and Sequencing:-

The polymerase chain reaction (PCR) primers were designed by using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome>, accessed 14 September 2015).

Table 1 Primer details

Gene	Primer Sequence	Product Length	Reference
Mt Control region	MITOCF 5′ - GGCTTGAAAAGCCATTGTTG -3′	~510 bp	Khaliq *et al*. 2011
	MITOCR 5′ - CCCCAAAGAGAAAAGGAACC -3′		

The sequences of these primers (Table 1) are derived of GenBank *Francolinus pintadeanus* mitochondrion sequence NC011817 (Shen et al., 2009). The map position of these sequences on mitochondrion is 16,739 to 16,758 (forward) and 668 to 649 (reverse) in the numbering of the original Desjardins and Morais (1990) chicken sequence (GenBank accession X52392.1). Therefore, these PCR conditions standardized here amplified a fragment including the first half of the CR, according to Desjardins and Morais (1990).

In the final PCR master mix (Table 2) 10-30 ng of isolated DNA was used with the primer concentration 0.1 pmole/µl for each primer. The PCR was carried out in a Gradient thermal cycler (Techne). The Taq polymerase (Genei, 1 unit/µl) was used in the amplifications (1 unit for 25 µl final volume reaction mixture) following the manufacturer's instructions and using the amplification buffer (10X) supplied with the taq polymerase.

Table 2 PCR master mixture

Components	Final Concentration in single PCR Tube	Volume (µl)
Autoclaved distilled water		17.0µl
Taq Assay Buffer (10X) with $MgCl_2$	1X	2.5µl
dNTPs Mix (10mM each)	2.5mM	0.5µl
Primer (10 pmole/µl)	0.1 pmole/µl	0.5µl F 0.5µl R
Taq polymerase (1 U/µl)	1 U/µl	1.0µl
DNA Template	10-30ng	3.0µl
Total		**25.0µl**

The PCR conditions standardised were: 94 °C for 5 min followed by 35cycles of 94°C for 1 min, 55°C for 30s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

Table 3 Thermal Cycle Conditions

Cycle	Denaturation		Annealing		Extension	
	Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
First Cycle	Initial denaturation 94°C for 5 min					
35	94°C	1 min		30 sec	72°C	1 min
Cycles			55°C			
Last cycle	Final extension 72°C for 5 min					
Hold	4°C					

The PCR products were visualized on 1.5% agarose gels and the most intense products were selected for sequencing. Products were labelled with the Big-Dye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc., Foster City, California, USA) and sequenced bi-directionally using an ABI 3730 capillary sequencer following the manufacturer's instructions.

Sequence Analysis:-

Sequences of all the individuals were separately aligned using the program Clustal X 2.0 (Larkin et al., 2007). Length differences were resolved by inserting alignment gaps and positions that could not be aligned unambiguously were excluded. The degree of sequence disparity was calculated by averaging pair-wise comparisons of sequence difference across all individuals. Overall Base composition, number of transition and transversion from aligned sequences and Pair-wise evolutionary distance among haplotypes was determined using Molecular Evolutionary Genetic Analysis (MEGA) version 6.0 (Tamura et al., 2013).

Results and discussion:-

The amplified sequences were on average 475-648 in length. NUMTs (Nuclear DNA Sequences originating from mtDNA sequences) were not sequenced. In addition, because of the higher copy number of mitochondrial DNA, some studies have shown that NUMTs are detected though in a very small percentage. Moreover when detected, NUMTs regularly show indels or diagnostic mutations so as to reveal their presence, which was not observed in present analysis. In the present study, we detected no signs of pseudogenes. Further, this mitochondrial region analysis revealed overall average nucleotide frequencies A = 24.54%, T/U = 30.03%, C = 31.06%, and G = 14.37%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -1015.936. The analysis involved 12 nucleotide sequences. There were a total of 648 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0 (Tamura et al., 2013).

Table 4 Maximum Likelihood Estimate of Substitution Matrix

	A	**T/U**	**C**	**G**
A	-	*4.43*	*4.59*	**5.76**
T/U	*3.62*	-	**27.90**	*2.12*
C	*3.62*	**26.97**	-	*2.12*
G	**9.84**	*4.43*	*4.59*	-

Table 4 shows maximum likelihood estimate of substitution matrix for all sequences. Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei model (Tamura and Nei, 1993). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in *italics*. Relative values of instantaneous *r* were considered when evaluating them. For simplicity, sum of *r* values is made equal to 100.

Maximum Likelihood Estimate of Transition/Transversion Bias:-

The estimated Transition/Transversion bias (*R*) calculated was 2.79. Substitution pattern and rates were estimated under the Tamura-Nei model (1993). The nucleotide frequencies are A = 24.54%, T/U = 30.03%, C = 31.06%, and G = 14.37%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -1015.936. The analysis involved 12 nucleotide sequences. There were a total of 648 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0. The transitional bias suggests that this is a recently evolved group or slowly evolving genes. A transition bias in these genes means that there are few multiple substitutions and that the data therefore have phylogenetic signal. Overall the lower rate of transversions should lead to better resolution of deep divergence events because of low saturation effects.

Table 5 Base composition (%) for mitochondrial D loop region of Black francolin

Sample	T(U)	C	A	G	Total
Site1A4	30.8	31.0	24.0	14.2	626.0
Site1B4	30.7	30.4	23.9	15.0	648.0
Site1C4	30.9	30.7	23.8	14.6	635.0
Site2A6	29.6	31.1	24.9	14.4	598.0
Site2B6	29.5	31.2	24.9	14.4	599.0
Site2C6	29.6	30.5	25.5	14.4	597.0
Site3A8	30.2	31.3	24.1	14.4	623.0
Site3B8	30.3	31.1	24.2	14.5	621.0
Site4A11	29.7	31.6	24.6	14.1	610.0
Site4B11	29.6	31.5	24.7	14.2	604.0
Site4C11	29.3	31.6	25.1	14.0	594.0
Site5A13	29.9	30.8	25.2	14.1	588.0
Avg.	30.0	31.1	24.5	14.4	611.9

Overall estimates of evolutionary divergence between sequences was also calculated. The number of base substitutions per site from between sequences are shown in Table 6. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 572 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0

Table 6 Overall estimates of evolutionary divergence between sequences.

	Site1 A4	**Site1 B4**	**Site1 C4**	**Site2 A6**	**Site2 B6**	**Site2 C6**	**Site3 A8**	**Site3 B8**	**Site4A 11**	**Site4B 11**	**Site4C 11**	**Site5A 13**
Site1A 4												
Site1B 4	0.004											
Site1C 4	0.009	0.009										
Site2A 6	0.004	0.002	0.009									
Site2B 6	0.004	0.002	0.009	0.000								
Site2C 6	0.005	0.002	0.011	0.004	0.004							
Site3A 8	0.005	0.007	0.014	0.005	0.005	0.009						
Site3B 8	0.007	0.005	0.005	0.004	0.004	0.007	0.009					
Site4A 11	0.009	0.009	0.014	0.011	0.011	0.011	0.012	0.014				
Site4B 11	0.005	0.005	0.014	0.007	0.007	0.007	0.002	0.011	0.011			
Site4C 11	0.005	0.005	0.004	0.005	0.005	0.007	0.011	0.002	0.014	0.011		
Site5A 13	0.005	0.007	0.007	0.005	0.005	0.009	0.007	0.002	0.012	0.009	0.004	

The overall estimates of average evolutionary divergence for all sequence pairs was 0.007. The number of base substitutions per site from averaging over all sequence pairs are shown. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 572 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0

Table 7 Results from Tajima's Neutrality Test

m	S	p_s	Θ	π	D
12	12	0.020979	0.006947	0.006914	-0.020220

m = number of sequences, n = total number of sites, S = Number of segregating sites, $p_s = S/n$, $\Theta = p_s/a_1$, π = nucleotide diversity, and D is the Tajima test statistic (Tajima 1989)

Table 7 shows the results for Tajima's test of neutrality (Tajima, 1989) which compares the number of segregating sites per site with the nucleotide diversity. (A site is considered segregating if, in a comparison of *m* sequences, there are two or more nucleotides at that site; nucleotide diversity is defined as the average number of nucleotide differences per site between two sequences). If all the alleles are selectively neutral, then the product *4Nv* (where *N* is the effective population size and *v* is the mutation rate per site) can be estimated in different ways and the difference in the estimate obtained provides an indication of non-neutral evolution. Tajima (1989) showed that if the population goes through a bottleneck, *D* can be significantly positive or negative depending on the population history.

Conclusion:-

The study provides a base line data on mitochondrial control region of Asian Black Francolin (*Francolinus francolinus asiae*) from Western Himalaya which would be the basis for a better understanding of an important group of game birds and for future study on them related to population and conservation genetics. In future, similar studies should be carried out so as to detect variation in population structure based on molecular markers. Further the present study on control region from one of the francolin species could be utilised for comparative studies in future with other species and subspecies of this genus and even other genera.

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