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Mitochondrial D-loop based genetic characterization of two Francolin species from a part of Himalayan foothills

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ABSTRACT

Genus *Francolinus* under the family Phasianideae of order Galliformes are important group of birds and among the largest genera in the class Aves, distributed throughout the world and occupying different habitats. This group has several IUCN red listed species undergoing population fragmentation, decline and habitat loss. So far only two species of genus *Francolinus viz*., Asian Black francolin (*Francolinus francolinus asiae*) and the North Indian Grey francolin (*Francolinus pondicerianus interpositus*) have been reported from Uttarakhand. Here we have characterized genetic-make up of these francolins using mitochondrial DNA marker i.e. D-loop or Control region. The genetic polymorphism pattern of the mitochondrial control region in Black and grey francolin, is described for the North Western Himalayan population in order to get some base line data about genetic diversity, possible population structure and demographic dynamics.

Key words: Galliformes, Uttarakhand, mitochondrial DNA, control region, genetic diversity

INTRODUCTION

Francolinus is the largest genus of the family Phasianidae of the order Galliformes (Morony *et al*., 1975) which includes ecologically diverse group of birds represented by one or more species in almost all biomes of the world, except the polar region (McGowan *et al*., 1995). The genus *Francolinus* was first described by Stephens (1819). These relatively smaller Galliformes have around 23 species listed as endangered and 6 as critically endangered in the IUCN red list (The World Conservation Union, 2012). The population of various species in this group is getting fragmented and declining within their distributional range due to predation, hunting, poaching and loss of food due to habitat destruction and uncontrolled forest fire.

Francolins are 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 (Johnsgard, 1988, del Hoyo *et al*., 1994, Madge and McGowan, 2002). Though they are difficult to separate from partridges due to similar looks; partridges are medium sized game birds (Crowe *et al.,* 1992) while francolins have slimmer body and relatively upright body posture (Crowe and Little, 2004). Francolins are also characterized by having 14 tail feathers that are presumably moulted centrifugally. This genus includes forty one species of which only five are restricted to Asia while remaining are restricted to Africa. Five species are found in India: Painted francolin *Francolinus pictus,* Chinese francolin *F. pintadeanus*, Grey francolin *F. pondicerianus*, Swamp francolin *F. gularis* and Black francolin *F. francolinus* (Grimmett *et al*., 1999, Ali and Ripley, 2001). Two species found in Uttarakhand under the genus francolinus are Asian black francolin (*Francolinus francolinus*), 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 Ripley, 1980). It has been reported to occur in the scrub habitats, having plenty of low shrubs and tall grasses and wetland (Khan, 1989, Roberts, 1991) or xerophytic vegetation in Sri Lanka (Wijeyamohan *et al*., 2003). In India black francolin is a resident and common in the lower Himalayas and its range extends up to 2100m inhabiting cultivated areas, tall grasses and scrub requires good ground cover, especially near the water resources, present in high population densities in different ecological zones of the country (Khan and Garson, 1997, Ali and Ripley, 2001). The grey francolin is found from Sri Lanka across the Indian subcontinent to the foothills of the Himalayas, East to Bengal and West to eastern and southern Pakistan and across South Iran to the Persian Gulf. The North Indian grey francolin *(Francolinus pondicerianus interpositus)* has a wide distribution range and is better adapted to sustain arid conditions, living under sparse growth of vegetation (Ali and Ripley, 1983, Roberts, 1991, Fuller *et al*., 2000). In India it is rarely found above an altitude of 500 m and is a

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common breeding resident game bird. In Uttarakhand the North Indian Grey francolin is observed in terai, lower ranges of Shiwaliks and has never been sighted in Chir Pine or lower temperate regions (D.K. Sharma and Suman Negi, Personal Communication).

Francolins though non-migratory, occupy diverse habitat types at different altitudinal range (Bump and Bump, 1964, Johnsgard, 1988). Varying altitude, topography and landscape characteristics influences the availability of suitable habitat and dispersal of a species and thus influencing the genetic structure among populations. Recent genetic advances allow better assessment of the role of ecological and evolutionary processes in determining the dynamics of genetic diversity in natural populations. The mtDNA continues to provide perhaps the best information for linking genetic change to organismal change, which is a prerequisite for understanding the genetic basis of evolutionary change at the organismal and population levels. Mitochondrial D-loop (or control region) has been extensively usually used in ecological, evolutionary, and genetic approaches to analyse genetic structure. Forcina *et al*. (2012) worked out the fundamental questions regarding the evolution of the Asian francolins by sequencing the entire control region of five Asian francolin species.

No genetic study has been conducted so far from the Himalayan region. The present study is in continuation to the earlier work from our group on the breeding and habitat ecology of the *Francolinus* species from North Western Himalayan region. Here we have described the genetic polymorphism pattern of the mitochondrial control region in Black and grey francolin, first time from this region in order to get some base line data about genetic diversity, possible population structure and demographic dynamics for the North Western Himalayan population.

MATERIALS AND METHODS

Study area

Uttarakhand falls under the North-western Himalayan region and located between 28º43'-31º27' N latitudes and 77º34'-81º02' E longitudes. It is divided into two zones non-montane and montane physiographic zones (Mani, 1974). Non-montane zone includes Bhabhar, the foothills of the Himalayas and Tarai which is situated below the Bhabhar. Montane zone includes Sub-Himalayas possesses the least of Himalayan features. It consists of two zones, the Shivaliks which is youngest of the Himalayan ranges and the Doon, flat longitudinal structural valleys with altitudes of 300 to 1000m. For this study, both species were sampled up to 500m as Grey francolin in this region is rarely found above that altitude and has been only observed in terai, lower ranges of Shiwaliks.

Study organism

Black francolin (*Francolinus francolinus asiae*) has average body length of 31-36 cm and 227-566 gm average weight with clear sexual dichromatism. Male has 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 (Ali and Ripley, 1983).

Grey francolin (*Francolinus pondicerianus interpositus*) is slightly larger in size than the Black francolin (del Hoyo *et al*., 1994). Both sexes look similar, but males are slightly larger and have sharp spurs, up to two on the legs while females usually lack them. Under parts are pale buff and rufous, narrowly cross-barred on foreneck and upper breast and finely vermiculated on abdomen and flanks with black. It has a prominent yellowish rufous throat patch enclosed with a black spotting loop, chestnut, grey-brown and dark brown barring to upper parts and chestnut on tail in flight (Ali and Ripley, 1983).

Sample collection

Preliminary surveys were conducted along altitudinal gradient up to 500m asl to understand the distributional pattern of the Black francolin and Grey francolin. The most preferred habitats of the population were also assessed. Trails were walked along the altitudinal gradients and subsequently samples were collected. Fresh feathers were collected from the study sites found separated from the body during the dust bathing behaviour of francolin. During the middle of the day black francolin and grey francolin rest under the bushes and at the same time they often perform dust bathing leading to fall of feathers from the body. These feathers were collected easily from the roosting ground. Liver and muscle tissue samples were only collected in those cases where the animal was found accidentally dead during field surveys. Tissue samples were placed in absolute alcohol at -20ºC for long term storage.

DNA Extraction

DNA from feathers and tissue was extracted using Chelax and QIAgen DNAeasy® Tissue Kit for non invasive isolation of DNA using feathers in most of the cases. 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 primers MITOCF 5′ - GGCTTGAAAAGCCATTGTTG -3′ and MITOCR 5′ - CCCCAAAGAGAAAAGGAACC -3′ (Khaliq *et al*., 2011) were used in this study. In the final PCR master mix 10-30ng 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, UK). 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. 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. The PCR products were visualized on 1.5% agarose gels and the most intense products were selected for sequencing. Products

Table 1. The vegetation composition at the sampled sites

Tree	Shrubs	Herbs	Grasses
Toona ciliate	*Lantana* spp	*Agertina adenophora*	*Cynodon dactylon*
Melia azaderach	*Rosa brunonii*	*Leucas lanata*	*Imperata cylindrical*
Bombax ceiba	*Carissa carandas*	*Bidens biternata*	*Cypercus rotundus*
Morus alba	*Acacia nilotica*	*Centella asiatica.*	*Fumeria indica*
Ficus oligodon	*Agave spp.*	*Solanum nigram*	*Cyperus spp.*
Dalbergia sissoo	*Colebrookia oppositifolia*	*Echinops echinativ*	*Asparagus adsendens*
Melia azederach	*Urtica dioica*	*Bidens pilosa .*	*Cannabis sativa*
Emblica officianalis	*Adhatoda vesica*	*Gloriosa superba*	*Euphorbia hirta*
	Datura stramonium	*Achyranthes aspera*	*Anagallis arvensis*
	Ricinus communis	*Aerva lanata*	
	Zizyphus vulgaris	*Solanunm nigrum*	
	Indigofera tinctoria	*Parthenium hysterophorus*	
	Crotolaria spp.		

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 (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 was determined by the Kimura 2-parameter (K2P) method using Molecular Evolutionary Genetic Analysis (MEGA) version 6.0 (Tamura *et al*., 2013). To estimate the number of polymorphic sites with state frequencies and expected diversity, the haplotype diversity (h), the mean number of pairwise differences (k) at haplotype level, the nucleotide diversity (π) and the haplotype frequencies. To track diversity in the populations, above basic diversity estimates values were calculated in DnaSP 5.10.1 (Librado and Rozas, 2009).

RESULTS AND DISCUSSION

Habitat and breeding preference are important constituents that determine the gene flow and genetic structure of a single species or a closely related species. These factors have cumulative effect on the phylogeography and determine diversity of genes accumulated by the species along the course of evolutionary history. Within their distributional range in subtropical tarai region in Uttarakhand the francolins occupies diverse type preferring different microhabitat components. The major plant species were identified from selected sites which found strongly associated with the species survival in the particular area (Table 1). At all the study sites it was observed that vegetation cover increases April onwards which provides cover, shelter and ample food in terms of insects and grass seeds for francolins. Increased vegetation cover also helps hide incubating female and guarding males from predators.

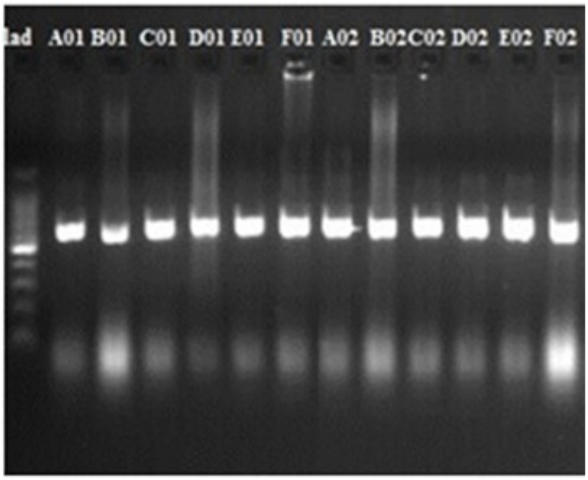

Figure 1. Amplified mitochondrial D-loop region of different samples for Black and Grey francolin

PCR amplification and sequencing

A total of 12 individuals (6 of each species) were used for amplification and partial sequence analysis of mitochondrial D-loop. Sequencing of the control region produced on an average around 578 nucleotide base pairs. Amplified products are shown in Figure 1. Simplicity and un-ambiguity were observed among the sequences of mitochondrial region. The quality of the chromatograms, lacking double peaks; together with the correct alignment of the sequences with the ones available for a closely related species in GenBank, led us to consider the amplification of possible nuclear copies of mitochondrial genes as a very unlikely event. All the sequences were

Table 2. Average Nucleotide composition for all samples analysed in the present study

	T(U)	C	A	G	Total
BF UK Kotdwar1	30.8	31.0	24.0	14.2	626.0
BF UK Kotdwar2	30.7	30.4	23.9	15.0	648.0
BF UP Bijnor1	30.9	30.7	23.8	14.6	635.0
BF UP Bijnor2	31.4	30.0	24.3	14.3	547.0
BF UP Saharanpur1	30.7	31.2	23.9	14.2	628.0
BF UP Saharanpur2	31.1	31.1	24.0	13.9	592.0
GF UK Haridwar1	29.9	28.7	26.2	15.2	401.0
GF UK Haridwar2	30.4	28.2	25.7	15.7	401.0
GF UP Najibabad1	31.5	31.2	22.1	15.2	619.0
GF UP Najibabad2	31.9	31.1	22.0	15.0	614.0
GF UK Rudrapur1	31.4	31.3	22.5	14.8	608.0
GF UK Rudrapur2	31.5	30.9	22.3	15.3	619.0
Avg.	31.1	30.6	23.6	14.7	578.2

submitted and GenBank accession numbers were obtained both for Black francolin (MN965509 to MN965514) and Grey francolin (MT310961 to MT310966) samples.

Genetic characterization

Nucleotide Frequencies

The mitochondrial Control region analysis revealed nucleotide frequencies as A=23.60%, G=14.70%, T=31.10%, C=30.60% for all the samples (Table 2).

Maximum Likelihood Estimate of Transition/ Transversion Bias

The estimated Transition/Transversion bias (*R*) is 2.45. 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 (Table 3). Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Relative values of instantaneous *r* were considered while evaluating. For simplicity, sum of *r* values is made equal to 100. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -896.615. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 393 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Table 3. Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	*4.23*	*4.23*	**11.79**
T/U	*3.91*	-	**17.83**	*1.93*
C	*3.91*	**17.82**	-	*1.93*
G	**23.95**	*4.23*	*4.23*	-

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.

Evolutionary Divergence

Evolutionary divergence based on the number of base substitutions per site from between sequences was also calculated (Table 4). Analyses were conducted using the Maximum Composite Likelihood model (Tamura, Nei and Kumar, 2004). The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 393 positions in the final dataset.

A well evident divergence pattern was observed among all the sequence analysed for two francolin species. Individuals of black francolin significantly distanced from grey francolin. Inter-specific genetic diversity ranged from 3.6 to 4.3% between the samples of two species. However, low Intra-specific genetic distance ranging from 0.1 to 0.9% was observed.

Genetic Structure

For all the individuals studied for black and grey francolin, at the identified polymorphic sites, other diversity indices were also calculated, as shown in Table 5. There were no records of either insertions or deletions. Populations of both species were characterized by low values of nucleotide diversity.

CONCLUSION

The genetic-make up of two francolins species, resident in Uttarakhand and adjoining areas was characterized using mitochondrial DNA marker i.e. D-loop (or control region). Many similar studies worldwide have focused on the genetic structure of different populations of this

Table 4. Estimates of Evolutionary Divergence between Sequences

	1	2	3	4	5	6	7	8	9	10	11	12
BF_UK_Kotdwar1												
BF_UK_Kotdwar2	0.001											
BF_UP_Bijnor1	0.004	0.004										
BF_UP_Bijnor2	0.004	0.004	0.001									
BF_UP_Saharanpur1	0.001	0.001	0.003	0.004								
BF_UP_Saharanpur2	0.004	0.004	0.002	0.001	0.005							
GF_UK_Haridwar1	0.038	0.039	0.037	0.036	0.039	0.036						
GF_UK_Haridwar2	0.042	0.043	0.041	0.040	0.043	0.040	0.003					
GF_UP_Najibabad1	0.038	0.039	0.037	0.036	0.039	0.036	0.002	0.005				
GF_UP_Najibabad2	0.040	0.041	0.039	0.038	0.041	0.038	0.004	0.006	0.001			
GF_UK_Rudrapur1	0.040	0.041	0.039	0.038	0.041	0.038	0.004	0.006	0.004	0.006		
GF_UK_Rudrapur2	0.041	0.042	0.040	0.039	0.042	0.039	0.006	0.009	0.005	0.006	0.004	

Table 5. The nucleotide diversity estimates from the number of polymorphic sites

	N	G+C	Pi	SD_{Pi}	k	Theta
Black francolin	6	0.443	0.009	0.001	4.8	0.007
Grey francolin	6	0.439	0.016	0.003	6.6	0.017

N=No. of Samples, G+C= GC content, Pi= Nucleotide diversity (per site), SD_{Pi} = Standard deviation of Pi, k= Average number of nucleotide differences, Theta (per site) from number of polymorphic sites

genus as this group of birds represent a valuable model for the purpose of comparison to other game species. However, no study has been conducted on genetic structure and speciation in these species so far from the Himalayan region. The present study was in continuation to our earlier work on the breeding and habitat ecology of the francolin species from North Western Himalayan region. The scientific data generated here is a baseline to explore further the gene flow, phylogeography and impact of habitat fragmentation in the distributional range of the species. The systematic long term data hence generated shall help wildlife conservationists, policy makers to design conservation startegies for ground dwelling indicator species like pheasant, partridges and quails and could be the basis for further decisions about population management and protection.

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