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Research Article

Genetic structure and diversity of Black Francolin in Uttarakhand, Western Himalaya, India

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Abstract

The present study evaluates the genetic diversity of black francolin (*Francolinus francolinus asiae*) in Uttarakhand on the basis of microsatellite loci. For this purpose, we examined five populations from three geographical zones of Uttarakhand, Western Himalaya. Microsatellite markers were polymorphic with the number of alleles per locus ranging from 4-21, effective number of alleles per locus from 1.34 to 4.93, the Polymorphic Information Content (PIC) value ranged from 0.22 to 0.85. The averaged observed heterozygosity across all loci was Ho=0.32±0.12 and averaged expected heterozygosity He=0.51±0.06 respectively. The genetic structure showed that there were two genetically distinct clusters. The Lesser Himalayan and Himalayan foothill population forming a single cluster and population of the Tarai region forming another cluster. The pairwise F_{ST} results showed a sizeable genetic difference between the population of higher and lower altitudes. The AMOVA showed that higher levels of variation were observed among individuals within populations (64.36%) and lower differentiation observed among populations (2.99%). Overall the populations of black francolin were genetically variable with high adaptive potential in Uttarakhand, Western Himalaya.

Keywords: Conservation, heterozygosity, homozygosity, genetic diversity, microsatellite markers, polymorphic sites

Introduction

The increased pressure of habitat fragmentation, overhunting and induced anthropogenic activity, put the number of species in threat. The Himalayan geographical region is facing the same. The protection of species requires a thorough understanding of species biology as well as of the level and distribution of genetic diversity (Neel andEllstrand 2003). The latter is an important factor for the adaptation and survival of the natural populations in the changing environment (Lande 1988; Landeand Shannon 1996). However current conservation practice focused on captive breeding, which reduces genetic diversity and this can severely affect the capability of a population to cope with the fluctuations in the environment (Landeand Shannon 1996, Frankham 2002, 2010).

The Himalayan ecosystem is a kind of sky island, where the distribution of species restricts to their geographical boundaries. This provides uniqueness in genetic diversity among the species. Galliformes are widely distributed in the entire Himalayan range. The black francolin is one of the key species of genus francolin with the presence of five of species and six sub species (Forcina*et al*. 2012). Black francolin (*Francolinus francolinus asiae*) widely distributed from Kashmir to West Bengal along with the foothills of central Nepal, Bihar, and thence through Punjab, Uttar Pradesh, Madhya Pradesh to the Chilka lake in Odisha (Ali and Ripley 1983, McGowan and Kirwan 2015). Although the Bird Life International (2016) listed black francolin is stable at global level, but

there were some reports indicate that its population is declining in many parts of its native range (Behbash *et al*. 2010, Riaz *et al*. 2011).

Despite habitat preference, feeding habit and population abundance (Negiand and Lakhera 2016, 2019), there is no significant study on genetic diversity of the black francolin in India. In Indian Himalayan region, there is a strong need for conservation programs based on genetic data. In the present work, our aim is to carry out the first in-depth genetic study of black francolin in the Garhwal Himalayan region to get useful information and offer logical management recommendations for the purpose of sustainable use and long-term protection of the species within an adaptive conservation framework. In this work, the genetic makeup of the black francolin resident in Western Himalaya was characterized using microsatellite (short tandem repeats, STR) DNA markers.

Material and Methods

Study Area

The Uttarakhand ($28^0$44' to $31^0$28'N and $77^0$35' to $81^0$01' E) encompasses 53,483 Km^2 and is situated in the Western Himalayan region of India. It is India 's youngest mountain state and according to biogeographical classification by the Wildlife Institute of India, the state comes into the Biogeographic Zones 2B Western Himalaya and 7B Shiwaliks (Rodgers *et al.* 2000). Uttarakhand is divided into five distinct topographic regions i) snow cover Trans Himalaya, ii) Sub Himalaya, iii) Lesser Himalaya, iv) Bhabar Himalayan foothills and v) Tarai. The black francolin is distributed from 100m to 2200m asl. So, to cover all representative topographic zones, five sites were selected. Two sites were selected from Lesser Himalaya (Rudraprayag and Jaunsar), one from Bhabar Himalayan Foothills (Kotdwar) and two from Tarai region (Haridwar and Udham Singh Nagar) (Fig. 1). A total 56 samples were collected from the period of 2013 to 2015.

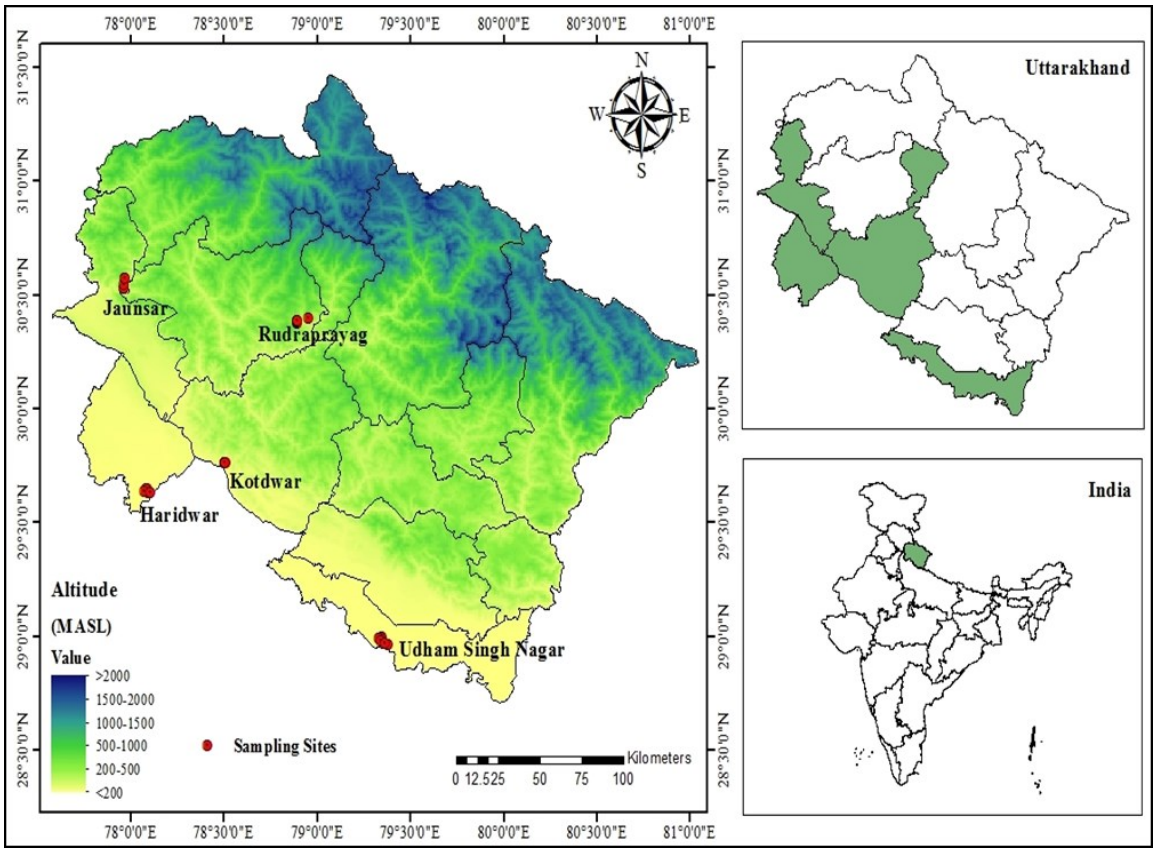

Figure 1. Different sampling sites of black francolin from Uttarakhand, India

DNA extraction and genotyping

A combination of the standard phenol-chloroform method was used for the extraction of DNA from tissues (Sambrook and Russell 2001) while a modified phenol-chloroform method (Barbanera *et al.* 2005) and Qiagen DNA isolation kit were also used for feathers. In view to examine the quality and concentration of extracted DNA, 2 µl of each DNA sample was loaded into a 0.8% agarose gel containing ethidium bromide. The concentration of DNA was visualized under UV transilluminator. A total of eight polymorphic cross-amplified microsatellite markers was standardized for the genetic analysis of black francolin (Negi and Lakhera, 2018). All individuals were genotyped with eight dinucleotide microsatellite loci (MCW81, MCW330, MCW183, MCW11, MCW206, 64D4, MCW104, MCW 145). The important details of the markers are given in Table1. PCR was performed in 25µl reaction mixture, containing 50-100ng DNA, 100ng of each primer, 2.5mM of each dNTPs, 10X Taq assay buffer and 3U Taq polymerase.

The amplification conditions were: 4 min initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at a specific temperature (Table 1) for 50 s and extension at 72°C for 1 min with a final extension at 72°C for 5 min. Subsequently, the PCR products were separated by capillary electrophoresis on analyzer ABI 3500XL (Applied Biosystems) and the allelic sizes of the fragments were estimated based on fluorescently labeled forward primers (FAM, and HEX) using the GENEMAPPER 3.7 (Applied Biosystems, USA) and Peak analyzer 1.0.

Table 1. Description of microsatellite loci used in the study

Loci	Fluorescent Label	5'-3' Sequence	Amplicon size	Annealing temperature T_A (^{0}C)
MCW 81	FAM	**F** GTTGCTGAGAGCCTGGTGCAG **R**ACACCCTGTATGTGGAATTACTTCTC	112-135bp	60^{0}C
MCW 206	HEX	**F** CTTGACAGTGATGCATTAAATG **R** ACATCTAGAATTGACTGTTCAC	221-249bp	50^{0}C
P6A1	FAM	**F** GATCTCACCAGTATGAGCTGC **R** TCTCACACTGTAACACAGTGC	190-234bp	52^{0}C
MCW 11	HEX	**F**GTTATATTTAATGTCCACTTGTCAATGATG **R** TAAACCACTTCACATGGAGCCT	96-120 bp	60^{0}C
MCW 330	HEX	**F**TGCTGGACCTCATCAGTCTGACAG **R**CAAACAAAATGTTCTCATAGAGTTCCTGC	256-300bp	55^{0}C
MCW 183	FAM	**F** GATCCCAGTGTCGAGTATCCGA **R** CTGAGATTTACTGGAGCCTGCC	296-326bp	55^{0}C
MCW 145	HEX	**F**CAATTTAACTTTATTCTCCAAATTTGGCT **R**GAGTAAACACAATGGCAACGGAAA	180-210bp	52^{0}C
MCW 104	FAM	**F**CTTTTTAGCACAACTCAAGCTGTGAG **R** CACAGACTTGCACAGCTGTGACC	210-225bp	55^{0}C

Data analysis

Genetic diversity and population differentiation: Allele frequencies and genetic diversity measures were calculated using GenAlEX 6.5 (Peakall and Smouse 2012) and FSTAT 2.9.4 (Goudet 1995). These measures included the number of alleles (Na), number of private alleles (Ne), expected heterozygosity or Gene Diversity (HE) and observed heterozygosity (HO). GENEPOP 4.1 (Rousset 2008) was used to infer possible departure from the Hardy-Weinberg Equilibrium (HWE) at each locus and evidence of linkage disequilibrium. Using allelic frequencies, Polymorphic Information Content (PIC), a measure of marker informativeness was calculated with CERVUS 3.0 (Kalinowski*et al*. 2007). Genetic differences between populations were analyzed with pairwise FST (Weir and Cockerham 1984). The significance of hierarchical analysis of molecular variance (AMOVA) was estimated within and between different populations with the software ARLEQUIN 3.5 (Excoffier and Lischer 2010).

Population structure

Population structure was assessed by the Bayesian model-based approach implemented in the STRUCTURE v.2.3 software (Pritchard *et al*. 2000) and Neighbor-Joining (NJ) phylogenetic tree (Saitou and Nei 1987). An individual-based Bayesian clustering procedure in STRUCTURE was run with values of K ranging from 1 to 10, with 200,000 burn-interactions and 1.0×10^5 Markov Chain Monte Carlo (MCMCs), with 10 independent runs for each K, using the admixture model with correlated allele frequencies. The most reliable value of K was chosen according to Evanno*et al*. 2005, implemented in STRUCTURE HARVESTER (Earl and VonHoldt 2012).

Analysis of Bottleneck: For analysis of the recent detection of bottlenecks in different demographic population software BOTTLENECK 1.202 (Piry*et al*. 1999) was used. In the present study, a one-tailed Wilcoxon signed-rank test of heterozygosity of excess (Luikart*et al*. 1998) detected under the Stepwise Mutation Model (SMM), the Infinite Allele Model (IAM) and the Two-Phase Model (TPM).

Results

Genetic Diversity: A total of 86 alleles were detected in the 56 individuals. The number of alleles detected per locus for the polymorphic loci ranged from 4 (locus MCW206) to 21 (locus MCW11) with an average of 10.75 ± 1.97 (Table 2). The effective number of alleles Ne per locus ranged from 1.34 (locus MCW81) to 4.93(locus MCW104) with an average of 2.56 ± 0.41. The observed and expected heterozygosity across the whole sample and all loci were HO=0.32 ± 0.12 and HE=0.51 ± 0.06 respectively. All eight microsatellite loci were highly polymorphic and the PIC value ranged from 0.22 (locus MCW81) to 0.85(locus MCW104) with an average of 0.58 ± 20 (Table 2).

Table 2. Genetic diversity parameters estimated at each of eight microsatellite loci across the all *F. f. asiae* population.

Locus	Allele (K)	PIC	N_E	H_O	H_E
MCW 81	08	0.223	1.343	0.134	0.231
MCW 206	04	0.315	1.450	0.040	0.266
P6A1	11	0.734	2.930	0.336	0.644
MCW 11	21	0.738	3.314	0.605	0.681
MCW 330	08	0.691	2.390	0.040	0.523
MCW 183	06	0.505	1.858	0.022	0.432
MCW 145	16	0.584	2.284	0.440	0.545
MCW 104	12	0.854	4.935	0.982	0.787
Mean	10.75±1.97	0.580±	2.563±0.41	0.325±0.12	0.514±0.06

K= number of alleles, PIC= Polymorphic Information Content, Ne= No of effective alleles, Ho= Observed heterozygosity, He= Expected heterozygosity, F= Fixation index

Overall, the high level of genetic diversity within population was observed (an average, Na=4.50 ± 0.37, Ne=2.56 ± 0.20, Ho=0.32 ± 0.05, He=0.51 ± 0.03, Appendix 1). Average alleles per locus (Na) were found highest in Rudraprayag (6.5±0.86) and lowest was observed in Haridwar (3.63 ± 0.62). The average number of effective alleles (Ne) ranges from 2.34 ± 0.39 for Haridwar to 3.02 ± 0.55 for the Rudraprayag population. The number of private alleles was observed minimum in Haridwar (0.13 ± 0.12) and maximum in Rudraprayag (2.63 ± 0.8) (Appendix 1).

The observed heterozygosity (Ho) was found higher in Udham Singh Nagar (0.44 ± 0.14) and lowest heterozygosity was found in Jaunsar (0.18 ± 0.13). The expected heterozygosity (HE) was found higher in Rudraprayag (0.59 ± 0.06) and lower in Haridwar (0.47 ± 0.08) (Appendix 1). The multilocus test showed that populations were deficient in observed heterozygotes and departure from the Hardy-Weinberg equilibrium (HWE) but the departure was not significant. Linkage disequilibrium was not detected between all pairs of investigated loci).

Population differentiation and Structure

The pairwise F_{ST} was observed significantly different among higher and lower altitude populations (Appendix 2). The maximum difference was observed between Jaunsar and Haridwar (F_{ST} = 0.15) followed by Jaunsar and UdhamSingh Nagar Population (F_{ST}= 0.14). The lowest F_{ST} differentiation was observed between Haridwar and Udham Singh Nagar population (0.03) and Jaunsur and Rudraprayag (0.03) (Appendix 2). The AMOVA showed that a higher level of variation was observed among individuals within populations (64.36%), sizeable variation among individuals between populations (32.63%) and lower differentiation observed among populations (2.99%) (Table 3).

Table 3. AMOVA design and results for different population of *F. f. asiae* inferred from eight microsatellite markers

Source of Variation	Sum of squares	Variance components	Percentage variation
Among populations	35.632	0.11702	2.99139
Among individuals within populations	321.948	2.51796	64.36899
Within individuals	71.500	1.27679	32.63962
Total	429.080	3.91177	

STRUCTURE was applied to the entire dataset. The result indicated a strong pattern of population differentiation with two population clusters (Fig. 2). The highest value of *ad hoc* quantity (Delta K) was observed 396.72 at K = 2. All samples from Haridwar and Udham Singh Nagar population were grouped into a single cluster (red cluster), while those samples from Jaunsar, Rudraprayag and Kotdwar population were grouped into another (green cluster). The samples from the Kotdwar region included individuals from both clusters (Fig. 1). Neighbor-Joining tree based on Nei genetic distance also detected two clustered population structures. The first cluster (Cluster A) comprised two populations of Lesser Himalaya (JaunsarandRudraprayag) and one from Bhabar (Kotdwar) and the second cluster (Cluster B) consisted population of Tarai (Haridwar and Udham Singh Nagar) (Fig. 3).

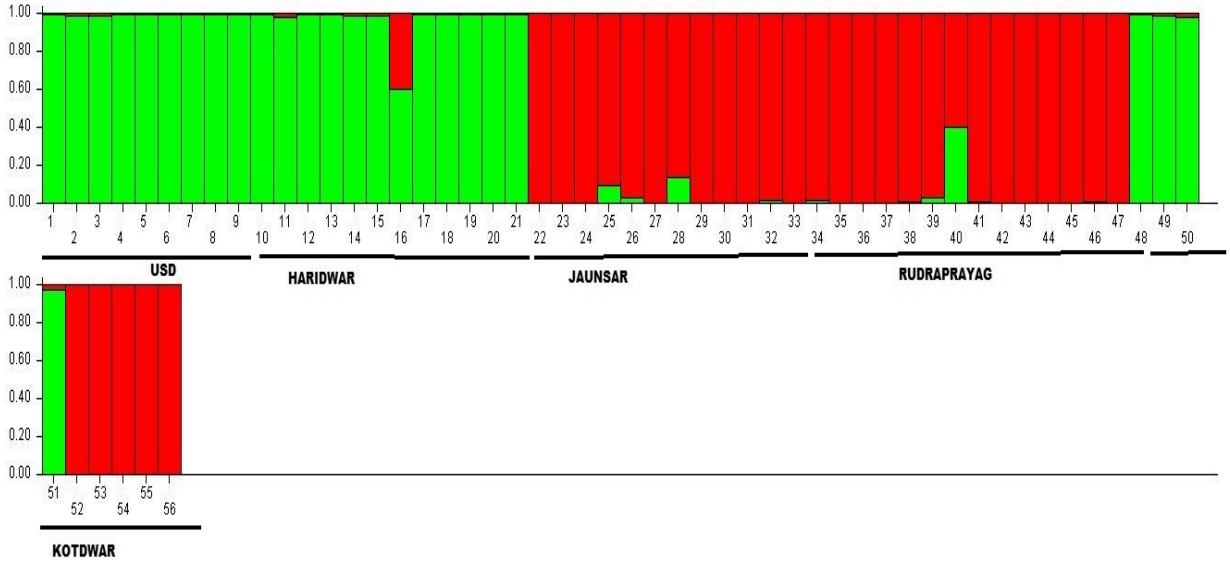

Figure 2. Bayesian admixture analysis of black francolin genotypes computed by STRUCTRE software, showing k=2

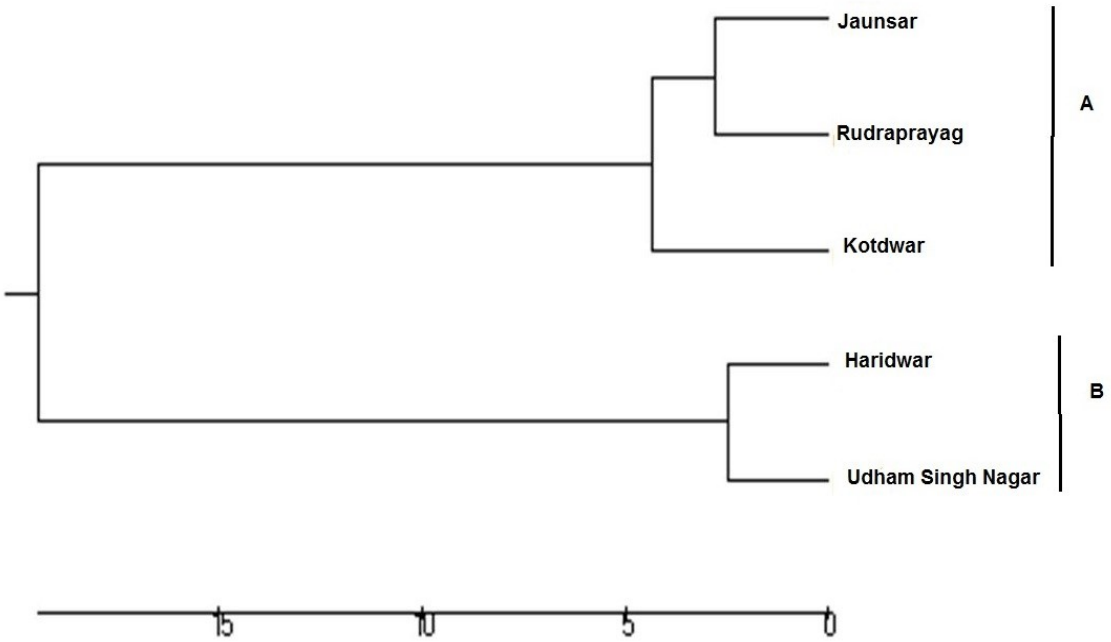

Figure 3. Neighbor Joining tree based on Nei genetic distance from microsatellite markers

Analysis of Bottleneck

Three mutation models (IAM, SMM, and TPM) were used under the Wilcoxon signed test for bottleneck assessment in five different populations of Black Francolin. Under the Wilcoxon rank test, the probability values of IAM, TPM, and SMM were not found to be significant ($p>0.05$). The results suggested that there is no recent reduction in the demographic population size of black francolin in the area of Uttarakhand (Table 4).

Table 4. Probability of heterozygosity excess according to Wilcoxon sign-rank test under three mutation model in *F. f. asiae* from different sampling sites

Population	IAM	SMM	TPM
Jaunsar	0.12500	0.42188	0.67969
Rudraprayag	0.14844	0.65625	0.90234
Kotdwar	0.09766	0.42188	0.80859
Haridwar	0.12500	0.27344	0.57813
USD	0.37109	0.62891	0.90234

IAM = Infinite allele model, SMM = Stepwise mutation model, TPM = Two phased model of mutation. P>0.05, no population has experienced bottleneck.

Discussion

This is the very first study on black francolin genetic structure based on microsatellite data from Uttarakhand. In the Garhwal region, the species is under threat due to rapid habitat fragmentations. Although Indian Himalayan is known for the rich diversity of Galliformes, most of them fall within threaten categories (Kaul 2007). Our previous study on habitat preference and distribution pattern of black francolin also suggests that population abundance is lower in the Tarai region due to higher anthropogenic pressure (Negi and Lakhera 2019). For considering this fact, we evaluated Lesser Himalayan, Bhabar Himalayan foothill and Tarai population of Uttarakhand on the basis of the genetic study.

For the present work, microsatellite markers were standardized from Red jungle fowl (*Gallus gallus)* and these markers were highly polymorphic in nature (Negi and Lakhera 2018). In Himalayan monal (*Lophophorus impejanus*), the number of alleles was 5 for MCW11, 5 for MCW 330, 4 for P6A1 and 7 for MCW81 (Thakur et *al*. 2011). In red jungle fowl, these were 7 for MCW11 (Mukesh*et al.* 2011). The selected markers for this study were highly polymorphic viz. 21 alleles for MCW11, 8 for MCW 330, 11 for P6A1, 8 for MCW81 with a mean value of 10.74 alleles per locus. The PIC value of markers is one of the informative parameters in population genetic analysis (Botstein *et al.* 1980). The PIC value of more than 0.50 indicates higher polymorphism in markers. In this study, all microsatellite loci proved highly polymorphic value (PIC=0.58, all loci). Thus, on the basis of the number of alleles and PIC values, most of the loci invested in this study showed high polymorphism.

Unfortunately, very few data are available for this bird to compare our genetic diversity results with others from previous studies based on microsatellite loci. Although to the best of our knowledge, a single study on genetic diversity of black francolin with microsatellite marker is reported by Forcina*et al.* 2014, who genotyped 77 samples from Cyprus with 9 microsatellite loci. They used cross-amplification loci from red-legged partridge (*Alectoris rufa*) and chicken but in their study, out of nine loci only four were found polymorphic so this study did not detect relevant inference of genetic diversity for comparing this present study. However, very low observed and expected heterozygosity emerged in that study (Nicosia, Ho 0.07 He 0.07; Paphos, Ho 0.14 He 0.12) for the *F. f. francolinus*. Their result showed almost 10 folds' lower heterozygosity in *F. f. francolinus* compared to *F. f. asiae*.

The number of alleles (Na), effective alleles (allelic richness) and private alleles were found high in the population of Rudraprayag, Jaunsar and Kotdwar region. The expected and observed heterozygosity were observed higher in Tarai population. This can suggest that maximum genetic diversity parameters were higher in Lesser Himalayan and foothill population as compare to Tarai populations. The Lesser

Himalayan region has its unique geography and is very rich in bird biodiversity (Kaul 2007). The impact of habitat fragmentation is lower as compared Bhabar foothills and Tarai area. The high number of private alleles in Rudraprayag supported the fact that local microclimate has an impact on population structure (Adam *et al*. 2016) and higher species richness of Galliformes also reported in this area (Kaul 2007).

On the other hand, the Tarai region is highly affected by anthropogenic pressures. Nevertheless, at the same time, the reason for higher heterozygosity in the Tarai population could be due to higher gene flow between Haridwar and Udham Singh Nagar due to a lack of physical barriers. It is also well known that landscape connectivity influences the gene flow among the populations (Vas *et al.* 2001, Coulon *et al.* 2004, 2006). Allele frequency distribution exhibited a mix of shared and private alleles, possibly indicating shared ancestral polymorphism and recent divergences. Sub structuring among groups, populations, and individuals was highly significant based on AMOVA. Differentiation among the populations was separated by much greater geographical distances. AMOVA data suggested that the genetic variability is higher in a distant population than nearby ones. It was suggested that the gene flow is mostly limited to fine spatial scales and higher in closer affinities. The N-J tree based on Nei genetic distances among populations provided a clear indication of genetic divergence between populations into two groups. Bayesian analysis of population structure gave the highest probability of the data with K = 2, also suggesting that birds from five different locations split into two distinct genetic clusters. Birds from Lesser Himalaya (Jaunsar, Rudraprayag) and Himalayan foothill (Kotdwar) were assigned to the same cluster and those from the Tarai region (Haridwar, Udham Singh Nagar) form another cluster.

The observed genetic differences between the two clusters were relatively significant. The absence of differences between populations within the clusters could be explained by two hypotheses. The first hypothesis suggests that geographically proximate populations are well connected by gene flow comparative to a larger distance population. The other hypothesis suggests that genetic diversity between populations is the result of historical gene flow processes which lead to the fragmentation of the larger population (Lowe *et al.* 2004). The first hypothesis can apply to geographically proximate Haridwar and Udham Singh Nagar populations with no geographical barrier hampering gene flow. The second hypothesis could explain the clustering between higher and lower altitude regions taking into account that physical barriers like mountain ridges, highlands, dense forest patches are expected to imply less genetic exchange. The population dispersed upstream from a lower altitude and the microclimatic conditions of Lesser Himalaya and Himalayan foothills supposedly shaped the genetic structure of the bird. So, these two clusters are geographically and ecologically distinct from each other. It is also suggested that individuals who are on the edge of regional boundaries can mediate gene flow among populations. Indeed, there is evidence of admixture, but that is limited to the Kotdwar population (foothill), where the geographical boundaries of Lesser Himalaya and the Tarai region are shallow. It can be hypothesized that the population of the Himalayan foothill act as a natural bridge or connecting Lesser Himalayan and the Tarai population. It is assumed that in past all these populations might be more connected but due to the increases in anthropogenic activities, they are now mostly isolated.

The pairwise F_{ST} and Nei genetic distance data suggested that there is a strong genetic differentiation between higher and lower altitude populations. This can be explained as black francolin (*F. f. asiae*) is a widely

distributed bird found from 100m to 2400m asl sharing the different types of ecological zone in Uttarakhand and falls within the different genetic cluster. High levels of genetic diversity in natural populations are coupled with a wide range of ecological types and niche variations (Prentice *et al.* 1995, Cao *et al.* 2010). Data also revealed that there is no bottleneck experienced by any population recently and no evidence of deviation from mutation drift equilibrium emerged.

Conclusion

The present study is a preliminary attempt to demonstrate the pattern of genetic diversity and genetic structure of game bird populations in a Himalayan ecosystem. The populations of black francolins were genetically variable with supposedly high adaptive potential in Uttarakhand. The observation of an overall high level of genetic diversity indicates that all populations are in stable condition at the present time. However, if individual genetic parameters are considered then the genetic diversity is lower in the populations of the Tarai region. In addition, the population abundance of black francolin in this area is under tremendous pressure due to anthropogenic interventions, unmanaged human settlement, rapid industrialization and development activities leading to habitat destruction and fragmentation. Thus, the major focus should be on the population of birds in the Tarai region from a conservation viewpoint. So, genetic insight on this bird should be useful in the formulation of effective conservation management strategies for black francolin and another coherent species in the Himalayan ecosystem.

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