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High Genetic Diversity Revealed by RAPD Markers in the Black Francolin (*Francolinus francolinus*, Galliformes) of Pakistan

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Abstract.- In this study random amplified polymorphic DNA (RAPD) markers were used to infer the genetic profile of the black francolin (*Francolinus francolinus*, Galliformes) (N = 23) collected in five Pakistani areas (Alipur, Bait Suvai, Chakwal, Haroonabad and Rakni). A total of 269 different RAPD bands were obtained using fifteen primers. Estimates of the percentage of overall polymorphism (87.79%), Shannon's diversity information index (H = 0.421) and Nei's average gene diversity index (I_N = 0.276) were comparatively high. UPGMA dendrogram based on Jaccard similarity index was constructed. Four clusters were found: one comprising two populations (Rakni and Haroonabad) and the other three including only one population. Our genetic findings represent a preliminary yet promising background for a better understanding of the adaptive strategy of black francolin, and will be soon implemented with deeper analysis based on mitochondrial and microsatellite DNA to help local conservation managers to plan an effective strategy to protect this species in the Pakistani range.

Key words: Conservation, DNA polymorphisms, *Francolinus*, genetic diversity, RAPD

INTRODUCTION

The *Francolinus* is the largest genus of the pheasant family, Phasianidae, of the order Galliformes (Morony *et al*., 1975). The black francolin (*Francolinus francolinus*) is one of the few francolins to have a range outside Africa. Six morphologic subspecies are recognized: *F. f. francolinus* (Cyprus, South Turkey, Middle East, Caspian region); *F. f. arabistanicus* (Iraq, South West Iran); *F. f. melanonotus* (East Nepal, West Bengal across North West Bangladesh to Assam and Manipur); *F. f. bogdanovi* (South East Iran, South and East Afghanistan, South West Pakistan); *F. f. henrici* (South to North Pakistan); *F. f. asiae* (extreme North East Pakistan, West Nepal, Gujarat to West Bengal in North India). The range of the species was formerly more extensive, but over-hunting has reduced its distribution and number (Madge and McGowan, 2002).

Within the Pakistani range, black francolins avoid very open or bare hilly country and are practically absent from the northern mountain regions and most of the Khaber Pakhtoonka and Baluchistan. They are very dependent for nesting upon good ground cover in the form of scrub jungle although they have adapted to shelter and forage in crops, particularly cotton and sugar cane. Relict francolin populations survive around irrigated forest plantations in the Punjab (particularly Lal Sohanra, Bahawalpur, Pirowal, Khanewal) and the river rainforest regions in Sindh (particularly in Badeen: Ali and Ripley, 1983; Grimett *et al*., 1998). In Pakistan the black francolin has considerably suffered from hunting and poaching pressure particularly around the Indus River and in the Sindh Province, which is used to harbour a handsome population of this species (Grimett *et al*., 2009). Especially given the sedentary habits of the species, the decrease in numbers due to hunting and poaching might have reduced the total genetic diversity of Pakistani black francolin (Roberts, 1992).

Very scarce genetic data are available in the literature about *Francolinus* genus (Bloomer and

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Crow, 1998; Kimball *et al*., 1999; Crowe *et al*., 2006; Shen *et al*., 2010). In this study, we utilized the RAPD (Random Amplified Polymorphic DNA) markers (Welsh and McClelland, 1990; Williams *et al*., 1990, Khaliq *et al*., 2010) in order to estimate the genetic diversity of the Pakistani populations/subspecies. Unlike traditional PCR, RAPDs do not require any specific knowledge of the target DNA sequence. Polymorphisms detected by this technique can be used as taxonomic markers in population studies of a wide variety of organisms as well as in study aiming at identifying hybrid organisms (*e.g.*, Barbanera *et al*., 2010a). By using several different primers, RAPD-PCR can potentially increase the resolution of genetic differences among individuals in population genetic studies (Apostol *et al*., 1996; Nybom and Bartish, 2000; Franck and Awadhesh, 2006).

We attempt to estimate the genetic population structure of *Francolinus francolinus* in the Pakistani range of the species' distribution. The identification of the forces responsible for the genetic make-up of a given species represents a very demanding task (Avise, 2004). However, the estimate of the genetic diversity is an inescapable prerequisite for formulating any sustainable conservation strategy (Schneller and Holderegger, 1996; Chen *et al*., 2006) aiming at preventing reduction of gene flow between populations (Francisco *et al*., 2007). Hopefully, along with the aim of identifying conservation units and increasing the extent and quality of data available on the distribution of the black francolin, we feel confident we will increase our ability to develop new management strategies (see Jiang *et al*., 2005; Groom *et al*., 2006).

MATERIALS AND METHODS

Study area and sample collection

Most birds were sampled across Punjab and Balouchistan. Collection sites included Bait Suvai 30°05'N, 70°43'E (River Indus, N=6), Alipur 29°23′N, 70°55'E (Punjab, N=4), Haroonabad 26°39′N, 74°21′E (Punjab, N=4), Rakni 30°35'N, 69°55'E (the only sampling site in Baluchistan, N=4), and Chakwal 32°56′N, 72°54′E (Punjab, N=5) (Fig. 1). In addition, two out of three morphological Pakistani subspecies are residing in the areas from which sampling have been done *F. f. henrici* (Bait Suvai, Alipur and Chakwal) *F. f. bogdanovi* (Rakni, Haroonabad).

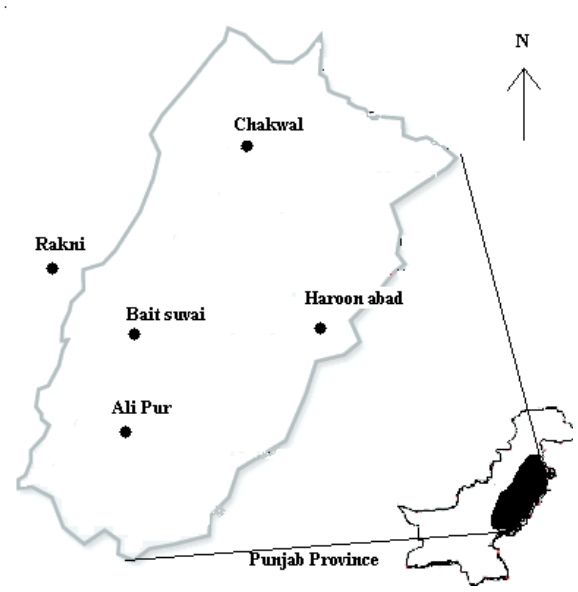

Fig. 1. Map showing the sampling sites in Pakistan.

DNA extraction

Only tail feathers were plucked and plunged down in 95% ethanol before they were stored at -20ºC. Total genomic DNA from individual feathers was extracted following Bello *et al*. (2001). A 0.5-1 cm long fragment from the base of the quill was used. Then, 500 µl of lysis buffer (50 mM Tris-HCl at pH 8, 20 mM EDTA at pH 8.2% SDS) was added together with 10 µl of proteinase K (final concentration, 175 µg/ml). Each sample was incubated at 55ºC overnight. The following day, a phenol:chloroform protocol for DNA extraction was employed (Sambrook *et al*., 1989). DNA concentration and purity were determined by spectrophotometry (Perklin Elmer Ltd., UK).

RAPD amplification

We screened 80 decanucleotide primers in order to select producing only clearly and reliably identifiable polymorphic bands (kits A, B, H and C from Genelink, USA). For each primer, we tested three times four individuals of each population in order to check for the reproducibility of the relative

RAPD banding profiles. Fifteen primers (Table I) yielded trustworthy band patterns. Hence, they were used to investigate all samples. PCR reactions (15 µl) were prepared as follows: 2.5mM $MgCl_2$, 10x PCR buffer 2.5mM of each dNTP, 50 ng/µl of each primer, 1.25 units of *Taq* DNA Polymerase (Fermentas, USA), and 50 ng of DNA template. Amplifications were carried out in a thermal cycler GeneAmp 9700 (Applied Biosystems, USA) with the following profile: 4 min at 94ºC, 45 cycles of 45s at 94ºC, 45 s at 37ºC and 1 min at 72ºC; then, a final extension of 10 min at 72ºC. Bands were separated by electrophoresis on 8% denaturing polyacrylamide gel and stained with $AgNO_3$ (Heukeshoven and Dernick, 1985; Budowle, 1991).

Table I.- Numbers and proportion of polymorphic bands generated by RAPD primers.

Primers	Sequence 5'-3'	Number of loci		Poly-morphism (P) (%)
		Total	Polymorphic	
GLH-01	GGTCGGAGAA	25	21	84
GLH-02	TCGGACGTGA	34	27	79.41
GLH-03	AGACGTCCAC	14	10	71.42
GLH-04	GGAAGTCGCC	16	14	87.5
GLH-05	AGTCGTCCCC	10	07	70
GLH-07	CTGCATCGTG	21	17	80.95
GLH-08	GAAACACCCC	23	14	60.86
GLH-09	TGTAGCTGGG	23	18	78.26
GLH-11	CTTCCGCAGT	20	19	95
GLH-12	ACGCGCATGT	17	11	64.70
GLH- 13	GACGCCACAC	22	19	86.36
GLH- 14	ACCAGGTTGG	21	18	85.71
GLH- 15	AATGGCGCAG	14	08	57.14
GLH- 16	TCTCAGCTGG	21	16	76.19
GLA- 14	TCTGTGCTGG	17	16	94.11
	Total	**269**	**235**	**87.79**

Data analysis

The presence/absence of each band was scored by analyzing the electrophoretic profile obtained of each specimen (Table I). The resulting matrix was imported into different programs for data elaboration. Each locus was treated as a two-allele system, with only one of the allele per locus being amplifiable by the PCR under the Hardy-Weinberg Equilibrium (Lynch and Milligan, 1994). The Nei's (1973) average gene diversity (I_N) and the Shannon Index (H) (Lewontin, 1972: $H = - \sum P_i \log_2 P_i$, where P_i is the frequency of a given RAPD band) were calculated using POPGENE (v. 1.31: Yeh *et al.*, 1999). The following calculations were carried out using the TFPGA software (v. 1.3: Miller, 1997) with Lynch and Milligan's (1994) correction: the estimation of the genetic polymorphism (P) and the Nei's (1978) unbiased genetic distance (D). The Principal Coordinate Analysis (PCoA) was carried out using the Euclidean distance matrix obtained in the MVSP software (v.3.1: Kovach, 2001). Finally, dendrograms were constructed using the Jaccard (J) coefficient and the UPGMA cluster analysis with the NTSYS-PC program (version 2, Rohlf, 1992).

RESULTS

A total of 296 clearly identifiable bands were obtained from 23 francolins: their size range was comprised between 130 and 1900 bp. Each primer produced 10-33 bands (with an average of 21.5) (Table I): the 89.6% was polymorphic (P). The genetic diversity was $I_N = 0.276$ and the Shannon's Index H= 0.421 (Table II). The effective number of alleles per loci at species level was A_e =1 .460, whereas the observed number of alleles per loci was A = 1.877 (Table II).

Table II.- Genetic variation.

	N_p	P	A	A_e	I_N	H
Overall	151	87.79	1.877	1.46	0.276	0.421

N_p, Number of polymorphic loci; P, Percentage of polymorphic loci; I_N, average gene diversity; H, Shannon Index; A, Observed number of allele per loci; A_e, Effective number of allele.

The smallest genetic distance (D) was observed between Rakni and Haroonabad birds (D = 0.016), whereas the largest one was observed between those from Rakni and Alipur (D = 0.210). In the dendrogram four large clusters were produced, which were further divided into smaller clusters: similarity coefficients ranged between 0.66 and 0.96 (Fig. 2). The cluster I comprises birds from Rakni and Haroonabad (Fig. 2). The birds belonging to the remaining three populations grouped together in other three clusters. The genetic distances among the four clusters ranged between 0.021 and 0.086 (Table III). The Principal Coordination Analysis showed that the individuals grouped together according to their locality except birds of Rakni and

Haroonabad (Fig. 3). Axis 1 extracted 14.03% of the total genetic variability while Axis 2 extracted the 10.80% (Fig. 3).

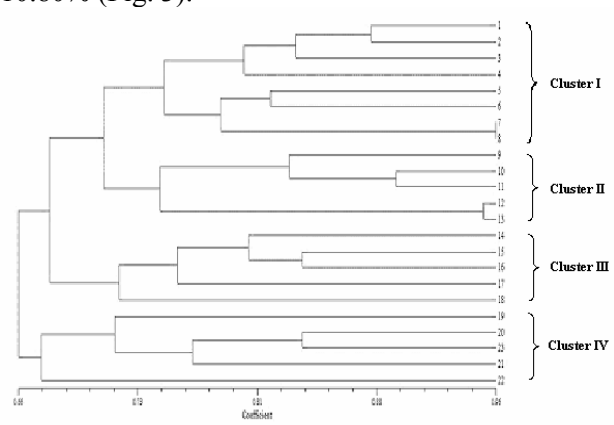

Fig. 2. Genetic similarity dendrogram generated with the Jaccard Coefficient and UPGMA method.

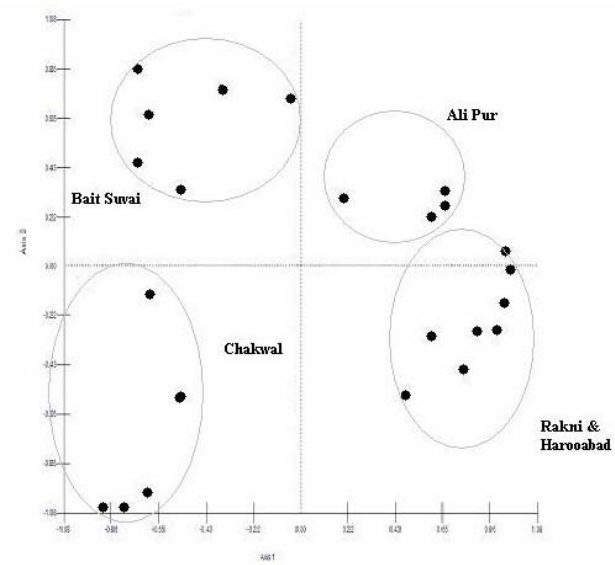

Fig. 3. Principal Coordinates Analysis of *Francolinus francolinus*: Axis 1 extracted 14.03% and Axis 2 extracted 10.80 of the variance.

Table III.- Genetic distances (UPGMA) between clusters.

	Cluster I	Cluster II	Cluster III	Cluster IV
Cluster I	*****			
Cluster II	0.037	*****		
Cluster III	0.065	0.028	*****	
Cluster IV	0.086	0.049	0.021	*****

DISCUSSION

In spite of its wide distribution and commercial value, the population genetic structure of the *Francolinus francolinus* is totally unknown in the whole range of the species. Until recently, only cladistic work dealing with the entire order of Galliformes adopting both molecular (Cytochrome-*b* gene) and morpho-behavioural approaches to reassess the overall phylogeny have been done (Bloomer and Crowe, 1998; Dyke *et al*., 2003; Crowe *et al*., 2006). So, this is the first study ever attempting to determine the population genetic structure of *F. francolinus* in Pakistan and in the entire species' range.

We used RAPD markers to investigate Pakistani black francolins. RAPD markers have gained widespread acceptance in genetic studies of many groups of organisms including bird species (Semenova *et al*., 1996; Giesel *et al*., 1997; Dolmatova *et al*., 2000; Muhammad *et al*., 2010). We examined eighty primers. Fifteen were selected as they exhibited reproducible and polymorphic output (Table I). The number of amplified bands varied from 10 to 33: each black francolin showed a unique RAPD banding profile. Furthermore, the large majority of primers that resulted in successful amplifications were polymorphic; hence, they proved to be very useful in resolving the genetic structure of the studied population.

In *F. francolinus*, the overall polymorphism at species level (P = 87.79%, Table I) was quite high, a result comparable with that obtained from the local population of the Manchurian pheasant while applying RAPD (*Phasianus colchicus*) (P = 79.4%) (Kulikova *et al*., 2002). Moreover, 81% and 86% of polymorphisms detected in the domestic mallard (*Anas platyrhynchos*) and in the spot billed duck (*Anas poecilorhyncha*) (Novikova *et al*., 2000), respectively. Different authors using RAPD markers have reported either low (red-cockaded woodpecker *Picoides borealis*, light-footed clapper rail, *Rallus longirostris levipes:* Bowditch *et al*., 1993; Haig *et al*., 1994; Nussar *et al*., 1996) or high (carrion *Corvus corone,* jungle crows C*orvus macrorhynchos*: Spiridonova *et al*., 2003) levels of genetic diversity in avian species. In our study the genetic diversity parameters levels were also very

high (I_N = 0.276 and H= 0.421 (Table II)). Like Spiridonova *et al*. (2003) this technique has enabled us to identify high levels of genetic diversity in *F. francolinus,* although the census of the species has decreased sharply throughout the Pakistan due to hunting pressure and habitat alteration.

The high genetic diversity of a population is related to many factors such as, among the others, out-breeding, habitat conditions, population size and introduction of birds from different sites or belonging to unidentified sub-species. In our case, we feel confident that introduction of birds from other sites is probably one of the main causes explaining the disclosed high genetic diversity. Allover Pakistan feudal breed these birds in captivity and time to time restock in their areas for hunting purposes. Majority of these captive birds are brought from other regions of the country. For instance, according to local people, the birds residing in Rakni were introduced from outside, i.e. probably from Haroonabad, as birds of these two localities grouped together (Figs. 2, 3). The distance between Rakni and Haroonabad is around 250 km. However, their genetic similarity suggested that birds from these localities have the same origin. Indeed, the genetic distance and Principal Coordinate Analysis also indicates that birds of Rakni and Haroonabad were genetically very similar (D = 0.016, Fig.2) and they likely belonged to same subspecies, namely *F. f. bogdanovi*. We guess that similar introductions might have occurred in other areas as well. Pakistani people like keeping black francolins at home as pets and participating to chirping competitions. There are freely and unchecked movement of black francolins throughout the Pakistan as people buy and sell these birds at a very high price (up to 300-400 US$ per bird). It is an establish fact that high level of genetic diversity means high fitness and high level of survival chances when sudden environmental changes do occur (Chen *et al*., 2006). Hence, the introduction of birds contributes to reinforce the local population gene pool by increasing the genetic diversity. However, translocations can contaminate the native genetic make-up of local populations/sub-species by introducing exotic genotypes and/or creating hybrids (see Barbanera *et al*., 2010b). In the *F. francolinus* the subspecies identification has not been yet sorted out conclusively, particularly in Pakistan where three of these subspecies co-occur. Hence, before introducing further birds this issue should be carefully addressed, as already done in similar species such as the chukar partridge (*Alectoris chukar*) (Tejedor *et al.,* 2007; Barbanera *et al.*, 2009)

Genetic distance is another parameter providing important insight into the differentiation among populations. The genetic distance between Rakni and Haroonabad was the smallest among all pairwise comparisons (D = 0.016, Fig.2). The largest value of the Nei's genetic distance (1978) was disclosed between birds from Alipur and Rakni (D = 0.21, Fig. 2). This suggests that birds of Alipur and Rakni belong to probably two different subspecies (*F. f. henrici*, Alipur and *F. f. bogdanovi*, Rakni). The estimates of genetic distances among populations/species reported for shiver ring-necked pheasant (*Phasianus colchicus*), subfamily *Anserinae* and in Bats, are very similar (range: 0.083 - 0.220) (Giesel *et al*., 1997; Baublys *et al*., 2002; Moreira and Moriella-Versute, 2006).

Overall, we found genetic evidence suggesting presence of at least two different subspecies, *i.e. F. f. henrici* and *F. f. bogdanovi*. This is evident from the UPGMA dendrogram as well (Fig. 2). The dendrogram showed that similarity coefficient varied from 0.80 to 0.97 (Fig. 2). Also the Principal Coordinate Analysis showed that, apart from Rakni and Haroonabad populations, all three remaining populations ordinate into different clusters (Fig. 3) indicating large genetic differentiation. All this suggests that all these birds have high degree of genetic diversity (probably due to restocking with birds from other areas) and moderate-to-high levels of genetic differentiation. The latter might be a consequence of reproductive isolation or presence of different sub-species, although any conclusion will be possibly drawn when a much larger sampling will be carried out.

CONCLUSIONS

The use of RAPD markers enabled us to pioneer in the determination of the population

genetic diversity and differentiation of the black francolin in Pakistan, suggesting the presence of at least two subspecies in five sampling sites. The genetic results here reported for the black francolin have relevant implication to plan conservation strategy for this species (Reed and Frankham, 2003; Hensen and Oberprieler, 2005). However, the conservation management of the black francolin is a hard task to be accomplished in Pakistan, as local people commercialise and thus translocate these birds, this being a handsome source of income. Birds from the Sindh Province are the most commercialised and restocked by local feudals after captive breeding, as a consequence, these birds risk to be distributed throughout the Pakistan. The survival of black francolin depends mainly on the protection of natural habitat and work with local communities to reduce over-hunting and poaching. The introduction of an effective law to put hunting under control as well as to avoid translocation of birds of unknown origin appears inescapable.

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