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Genetic diversity in see-see partridge (*Ammoperdix griseogularis*, Galliformes) populations from sub-Himalayan Mountain ranges of Pakistan

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ABSTRACT. We used Random Amplified Polymorphic DNA (RAPD) markers to investigate the genetic structure of two populations of see-see partridge (*Ammoperdix griseogularis*, Galliformes) from the Suleiman range, in the Pakistani Himalayan region. The see-see partridge is a vulnerable species with a distribution in the Middle East and central Asia. The percentage of polymorphic bands (94.05%), Shannon Index (H=0.455) and Nei's average gene diversity (I_N=0.298) *of A. griseogularis* at species level were rather high when compared with other avian species. 17% of polymorphic loci showed statistically significant differences in their allelic frequencies. The G_{ST} (Nei's coefficient of genetic variation) values indicated low levels of differentiation (G_{ST}=0.08). A genetic distance D of 0.05 indicated that both populations were to some degree in isolation but their differentiation was not significant. Overall, our genetic data can support action plans aiming to locally preserve differentiated genetic resources that, in the future, could potentially result in ecologically and behaviourally differentiated populations. In view of the rapid environmental changes that the Himalayan region has been experiencing in the last decade, this study could help in conservation plans.

KEY WORDS: *Ammoperdix griseogularis,* Genetic differentiation, Genetic variability, Himalayas, RAPD, Suleiman Mountain range

INTRODUCTION

The see-see partridge (*Ammoperdix griseogularis*, Phasianidae, Galliformes) is a bird species of dry and stony terrain listed as "vulnerable" (BIRDLIFE, 2004) This species has a huge distribution range, from southeast Turkey through Syria and Iraq to Iran and Pakistan (BAKER, 1924; ROBERTS, 1992; GRIMMETT et al., 1998). European populations suffer from demographic decline (BIRDLIFE, 2004). In Pakistan, see-see partridges are also present in Sindh Kohistan, the Punjab Salt Range and the North-Western Frontier Province (NWFP). However, in this latter region, the species is largely declining due to strong human persecution for hunting purposes (GRIMMETT et al., 2009). See-see partridge populations further colonize suitable terrains of the Balochistan, e.g. open, dry and hilly areas with limited agriculture. Birds are usually seen in pairs or, at the most, in flocks of two to four specimens. Yet, over fifty birds in a single flock have been counted in the Salt and Suleiman Ranges (KHALIQ, pers. obs., 2008), which are considered the strong hold of the species (ROBERTS, 1992) and present a well protected area due to tribal customs preventing easy access of outsiders.

The wide distribution range of the see-see partridge notwithstanding, no genetic data is available from the literature. To our knowledge, no work has been carried out to investigate the genetic structure of this species using DNA markers. Moreover, precise identification of the population genetic structure and possible natural population subdivisions is necessary to understand demographic and evolutionary patterns within/among populations, thus providing crucial information for future conservation plans (SCHAAL et al., 1991; WEBSTER et al., 2002; ZINK, 2004; HAIG et al., 2006; ALLENDORF & LUIKART, 2007). Hence, we employed Randomly Amplified Polymorphic DNA markers (RAPDs: WELSH & MCCLELLAND, 1990; WILLIAMS et al., 1990), a fingerprinting technique that has proved to be particularly valuable when DNA sequencing information is lacking.

MATERIALS AND METHODS

Sampling and DNA extraction

We collected 23 samples from two geographic populations of *Ammoperdix griseogularis* from the Suleiman range (Fig. 1) where their habitat is still intact and naturally protected due to the tribal restrictions mentioned above. Sampling was conducted between October 2006 and January 2008 (12 samples from the eastern population in October and November 2006, and 11 from the western population in November 2007 and January 2008). Each bird was captured at least four km away from other sampled birds to reduce the chance of sampling birds from the same covey. Only tail feathers were plucked, and plunged in 95% ethanol before they were stored at -50°C. Total genomic DNA from individual feathers was extracted following BELLO et al. (2001) from a fragment (0.5-1cm long) derived from the base of the quill. 500mL of lysis buffer (50mM Tris-HCl at pH 8, 20mM EDTA at pH 8, 2% SDS) was added, followed by 10mL proteinase K (final concentration, 175mg/mL). Each sample was incubated at 55ºC overnight. Then, a common phenol:chloroform protocol for DNA extraction was employed (SAMBROOK et al., 1989) and DNA concentration and purity were determined spectrophotometrically.

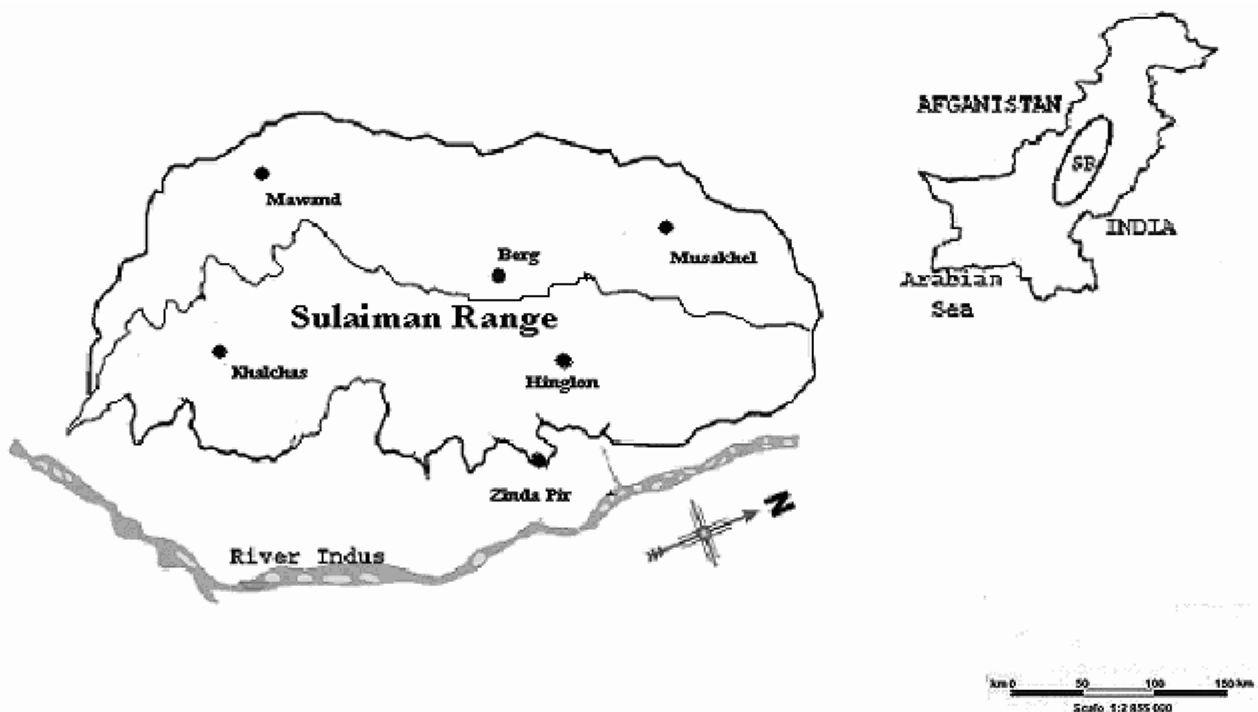

Fig. 1. – Map showing the geographical distribution of the western (Mawand, Berg and Musakhel) and eastern (Khalchas, Hinglon and Zinda Pir) populations of *Ammoperdix griseogularis* in the Himalayan mountains, Suleiman ranges, Pakistan.

RAPD amplification

The RAPD technique can be quickly and easily applied, requires only small amounts of DNA and allows detection of DNA polymorphisms reliably and inexpensively (Fritsch & Rieseberg, 1996; Harris, 1999). It has been successfully used in many genetic studies of avian population (e.g., Ball & Avise, 1992; Zink et al., 2000; Haig et al., 2004; Zink, 2004; Chan et al., 2008; Funk et al., 2008). For the Galliformes, RAPD markers have for example been successfully applied to detect hybridization among Mediterranean populations of different *Alectoris* partridges (Negro et al., 2001; Barbanera et al., 2005; 2009).

In order to select primers producing only clearly and reliably identifiable polymorphic bands, we applied 25 decanucleotide primers (kits A, B, H, from Genelink, USA; Table 3) to four individuals each from both populations. Each primer was applied thrice to check for reproducibility. Fifteen primers yielded trustworthy band patterns and were subsequently used to screen all samples. PCR reactions (15µL) were prepared as follows: 2.5mM $MgCl_2$, 10xPCR buffer 2.5mM of each dNTP 50ng/µL of each primer, 1.25 unit of *Taq*DNA Polymerase (Fermentas, USA), and 50ng of template DNA. Amplifications were carried out in a thermal cycler GeneAmp® 9700 (Applied Biosystems, USA) with the following thermal profile: 4min at 94ºC, 45 cycles of 45s at 94ºC, 45s at 36ºC and 1min at 72ºC; then, a final extension of 10min at 72ºC. Bands were separated by electrophoresis on 8% denaturing polyacrylamide gel and stained with $AgNO_3$ (Heukeshoven & Dernick, 1985; Budowle, 1991).

Data analysis

The presence or absence of each band was scored by analyzing the electrophoretic profiles obtained for all individuals using the 15 selected primers. The resulting matrix was imported into different programs for data elaboration. Each locus was treated as a two-allele system, with only one allele per locus being amplifiable by the PCR under the Hardy-Weinberg equilibrium (Lynch & Milligan, 1994). A G-test was applied to allele frequencies to check for homogeneity of allelic frequency distributions. In order to get a clearer inference from the G-test results, the statistically significant proportion of homogeneous alleles was estimated by dividing the number of total RAPD loci with statistically significant differences in allele frequencies (at least, 5%) by the total number of polymorphic loci. Nei's (1973) average gene diversity (I_N), Shannon Index (H) (Lewontin, 1972: H=-$\sum P_i \log_2 P_i$, where P_i is the frequency of a given RAPD band) and Nei's coefficient of gene differentiation (G_{ST}) between populations were calculated using Popgene v. 1.31 (Yeh et al., 1999). To compare the level of genetic diversity between populations, an analysis of variance for both the Shannon Index and the average gene diversity was carried out (randomized block design, two fixed factors: primer and I_N /H). Using the TFPGA software v. 1.3 (Miller, 1997) with Lynch & Milligan's (1994) correction, genetic variability and Nei's (1978) unbiased genetic distance (D) were estimated. The effective number of migrants per generation (N_{em}) was obtained from the formula N_{em}=0.25(1-G_{ST})/G_{ST} (Slatkin & Barton, 1989). Principal Coordinate Analysis (PCoA:) with Euclidean distance matrix was performed with the MVSP software

v.3.1 5 (KOVACH, 2001). Genetic similarity dendrograms were constructed with the Jaccard (J) coefficient and the UPGMA cluster analysis algorithm in the NTSYS-PC computer program (ROHLF, 1992).

TABLE 1

Numbers and proportion of polymorphic bands generated by RAPD primers

Primers	Number of loci		Polymorphism(%)
	Total	Polymorphic	
GLA-01	13	12	92.30
GLA-02	14	13	92.85
GLA-03	15	14	93.33
GLA-04	15	14	93.33
GLA-07	22	21	95.45
GLA-08	17	16	94.11
GLA-09	20	19	95
GLA-11	16	15	93.75
GLA-13	20	18	90
GLA-14	15	13	86.66
GLB-01	25	24	96
GLH-01	27	26	96.29
GLH-02	21	20	95.23
GLH-03	16	15	93.75
GLH-05	27	25	92.05
Total	**282**	**265**	**94.05**
Average	**18.8**	**17.66**	**-----------**

TABLE 2

Analysis of Variance for I_N/H within populations.

Source	Df	Sum of Squares	Mean Square	F
Population	1	0.138/0.061	0.028/0.012	3.810*/3.465*
Primer	14	0.203/0.098	0.014/0.007	1.997*/1.976*
Error	14	0.507/0.248	0.007/0.004	
Total	29	8.049/3.726		

TABLE 3

Genetic variation within population.

	Species	Population East / West
P	94.05%	85.71/78.57%
I_N	0.298	0.276/0.270
H	0.455	0.420/0.404
G_{ST}	---------	0.083
D	---------	0.05
N_{em}	---------	2.74
G-test	---------	17%

P=Percentage of polymorphic loci;
I_N=average gene diversity;
H=Shannon Index;
G_{ST}=Nei's coefficient of gene variation;
N_{em}=effective number of migrants per generation;
D=Nei's genetic distance,
G-Test=Homogeneity test

RESULTS

The selected 15 primers (Table 1) produced a total of 282 clearly identifiable bands with a size range of 125-1800bp in the two investigated populations. All primers produced polymorphic banding patterns varying from 13 to 27 bands (average 18.9 Table 1). The RAPD primers were found to be statistically significantly different in their ability to detect genetic diversity within populations (P<0.001, Table 2), thus providing sufficient reliability and effectiveness. The percentage of polymorphic bands (P) was 94.05% at species level (Table 1).The G-test showed that 17% of all polymorphic loci were statistically significantly different in their allelic frequencies between the two studied populations (Table 3). The intra-population variation was high in these two populations. In the eastern population, Nei's genetic diversity (I_N) was 0.276 and Shannon Index (H) was 0.420, whereas Nei's average genetic diversity (I_N) equalled 0.270 and the Shannon Index (H) was 0.404 in the western population (Table 3). Also the analyses of variance (ANOVA) for I_N and H showed a high genetic diversity in the studied populations (P<0.001, Table 2). The overall genetic diversity (I_N) at species level was also high; Nei's average genetic diversity reached 0.298 and Shannon Index (H) 0.455, respectively (Table 3). The number of migrants per generation was estimated as N_{em}=2.74 (Table 3). All parameters of genetic differentiation between the two populations showed low levels of differentiation, such as the coefficient of differentiation (G_{ST}) equalling 0.083 (Table 3) and the genetic distance (D) between these populations being D=0.05 (Table 3). In the Principal Coordinate Analysis, 25.26% of variation was represented by the two PCoA (Axis 1 and Axis 2) (Fig. 3).

DISCUSSION

This is the first study ever attempting to analyze the genetic structure of *Ammoperdix griseogularis* (see-see partridge) populations. Genetic data on this widely distributed species is very inadequate and only patchy information about the ecology of this species is available from the literature (BAKER, 1924; ROBERTS, 1992; GRIMMETT et al., 1998). Our analysis showed a high degree of genetic diversity in the species and at the population level. The intraspecific level of genetic polymorphism was around 94.05% (Table 1). This observed high level of genetic polymorphism was also supported by Nei's average genetic diversity (I_N=0.298, Table 3) and the Shannon Index (H=0.455, Table 3). Similar high levels of genetic polymorphism have been reported in other avian species such as Manchurian pheasant and Shiver ring-necked pheasant (GIESEL et al., 1997; KULIKOVA et al., 2002). The selected primers also detected a high degree of genetic polymorphism at the population level and the G-test showed that 17% of polymorphic loci differed significantly in their allelic frequencies (Table 3) between the two populations. In addition, the ANOVA for I_N and H supported high genetic diversity in these two populations (P<0.001, Table 2). This high degree of genetic diversity might be attributed to the sampling area providing undisturbed habitats in the Suleiman Range (due to the Tribal

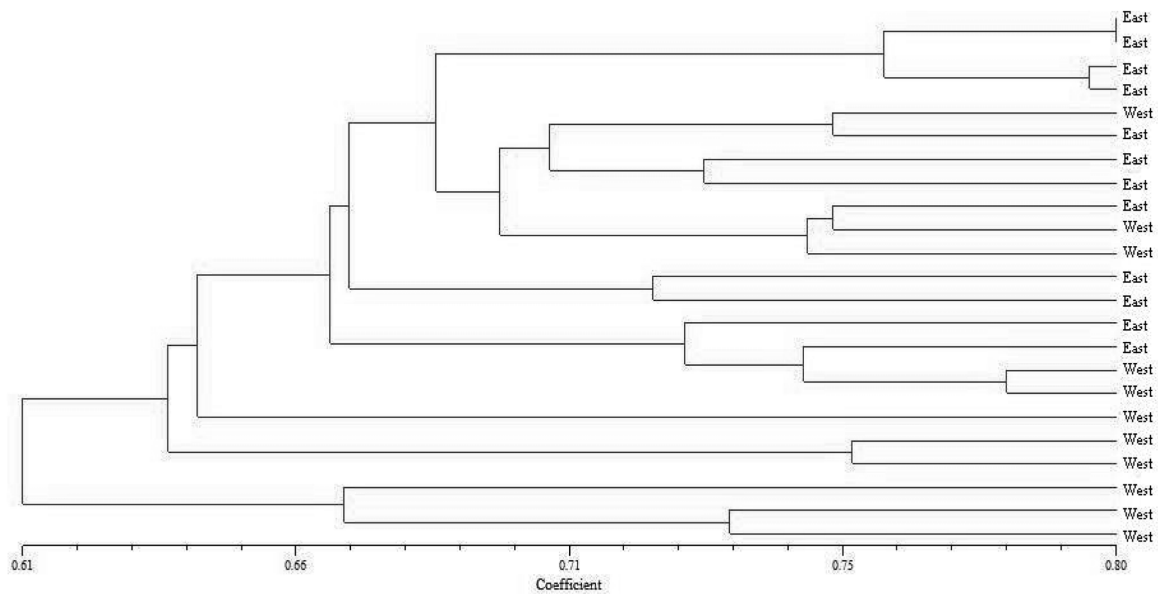

Fig. 2. – UPGMA dendrogram revealing genetic similarities between 23 *Ammoperdix griseogularis* genotypes based on RAPD amplification with 15 selected primers. The scale on bottom is NEI and LI's (1979) coefficient of genetic similarity.

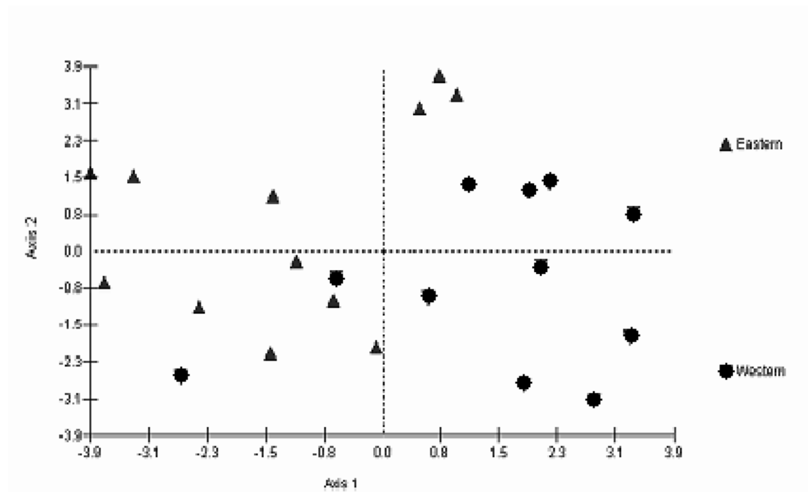

Fig. 3. – Principal Coordinates analysis of *Ammoperdix griseogularis*; Axis 1 comprised 14.99% and Axis 2 10.27% of the total variance.

restrictions, mentioned in the introduction). Morphologically, no distinct differences were observed in the specimens from the two studied populations. Specimens were similar in their size and plumage. The only visible difference between the two populations was the beak colour. Beaks from the western population had a yellowish tinge while birds from the eastern population displayed a red tinge. As far as the possibility of sub-species existence is concerned, no sub-species of *Ammoperdix griseogularis* (see-see partridge) have ever been reported except for a single study from 1950s by KOLEZ (1950) who suggested *Ammoperdix griseogularis Peraticus* as a new sub-species. This might just be a geographical morph as no other taxonomist or ornithologist ever acknowledged it.

In the past, RAPD identified high diversity at the population level in the Iberian eagle (*Aquilla adalberti*) where average genetic diversity was around 0.267 (PADILLA et al., 2000), similar to the results at the population level of our see-see partridge study (I_N=0.276 and H=0.420 for the eastern and I_N=0.270 and H=0.404 for the western population, respectively; see Table 3). Thus, the two populations investigated here showed similar levels of high genetic diversity.

ROBERTS (1992) and GRIMMETT et al. (1998) described the see-see partridge as a sedentary species that does not move long distances as this species prefers walking over flying, but our results do not support their observation. Despite the relatively large geographical distances of up to 150km between some sampling locations, genetic parameters suggested that the two see-see partridge populations were not significantly genetically differentiated. With a G_{ST} (coefficient of differentiation) of 0.083 (Table 3), results are comparable to the Light-footed clapper Rail (*Rallus longirostris levipes*) (NUSSER et al., 1996). Also the low genetic distance (D=0.05) between the two populations supported the low genetic differentiation between the two populations as in other avian species (Grasshopper sparrow (*Ammodramus savannarum*) with D ranging from 0.018 to 0.134 and an average 0.07; DOLMATOVA et al., 2000). The population similarity dendrogram revealed that the similarity coefficient varied from 0.61 to 0.81 (Fig. 2) suggesting that both populations are genetically similar. In the population dendrogram (Fig. 2) and the Principal coordinate analysis (Fig. 3), no separate clustering of the two populations was observed but specimens were rather scattered among clusters. This observation was also supported by the total number of migrants between these two populations that were N_{em}=2.74. This suggests that gene flow between these two populations is high (HEDRICK, 2000). In comparison with the Israeli chukar (*Alectoris chukar*, Phasianidae) populations, which showed very high numbers of migrants per generation N_{em}=6 and strong population structuring, (RANDI & ALKON, 1994), the value estimated in our study is low but high enough to keep the two populations from drifting apart (HEDRICK, 2000). So, on the basis of our genetic data, we do not consider this species as sedentary as previously reported.

CONCLUSIONS

Ammoperdix griseogularis showed a relatively high level of genetic diversity at the species and population levels, with substantial gene flow between populations thus resulting in low levels of genetic differentiation despite long distances between populations. Our study also illustrates that naturally preserved habitats in the Suleiman ranges could be an ideal conservation area because of their limited access due to tribal restrictions, thus providing a natural buffer zone for its wildlife, including *A. griseogularis*. Such areas are becoming very rare in the world due to human expansion and exploitation of natural resources. Our study also illustrates the need for more thorough genetic studies of this bird species with additional molecular markers and DNA sequence analysis to unravel its genetic structure in more detail.

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