Pakistan J. Zool., vol. 47(6), pp. 1739-1742, 2015.

Genetic Assessment of Some Pheasant Species from Dhodial Pheasantry Mansehra, Khyber Pakhtunkhwa, Pakistan

Lubna Shahi,[1] Inamullah,[2,*] Munawar Saleem Ahmad,[3] Ikram Muhammad[2] and Shahi Mulk[1]
[1]*Department of Zoology, Hazara University, Mansehra, Pakistan*
[2]*Department of Genetics, Hazara University, Mansehra, Pakistan*
[3]*Department of Zoology, University of Swabi, Swabi , Pakistan*

Abstract-. The present research was carried out to distinguish three species of pheasants at molecular level. Fourteen RAPD Primers were used over the nine samples of three pheasant species. On average 3.35 alleles were amplified and the average genetic distance estimated was 7%-63%. Nine samples of three species were clustered into two groups using dendrogram. Group A comprised of samples of Lady Amherst (*Chrysolophus amherstiae)* and Golden pheasant (*Chrysolophus pictus)* while group B comprised of Yellow Golden (*Chrysolophus pictus mut. luteus).* It is concluded that the Lady Amherst and Yellow Golden are most distantly related, so the crossing of these two species is recommended for creating maximum genetic diversity.

Key words: Genetic diversity, random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR), Lady Amherst pheasant.

INTRODUCTION

District Mansehra is located in the North East of Khyber Pakhtunkhwa. It is at an elevation of 975.36 meters (3200 feet) (Anonymous, 2009). Its total area is 4,579 Km^2. Its latitudes are 34º-14´ to 35º-11´ north and longitudes 72º-49´ to 74º-08´ east. On the north to Mansehra lies Kohistan and Battagram districts, on the east Muzaffarabad district of Azad Jammu and Kashmir, on the south Abbottabad and Haripur districts and on the west Shangla and Buner districts (Anonymous, 2007).

The flora of district Mansehra comprises Gulkhaira *(Althaea rosea)*, Sufed Musli *(Asparagus adscendens)*, Sumbal *(Berberis lyceum)*, Ispaghol *(Plantago major)*, Banafsha *(Viola odorata)*, Cha *(Camellia sinensis)*, Kutki *(Panicum miliare)* and Nera *(Skimea lyoela)*. Fauna including leopard *(Pathera pardus)*, jackal *(Canis aureus)*, wild goat *(Capra aegagrus)*, brown bear *(Ursus arctos)*, wolf *(Canis lupus)*, porcupine *(Hystrix indica)*, black bear *(Selenarctos thibetanus)*, monal *(Lophophorus impeganus)* and koklas *(Pucrasia macrolopha)* pheasant and other migratory birds are present that play a role in the long-distance spread of H5N1 (Anonymous, 2009; Cui *et al.*, 2014).

The town is prominent around the world for a conservation project for Pheasants**.** A Pheasantry facilitates for the breeding of pheasants is present. It was recognized to conserve ring necked pheasant and also other exotic pheasants. Currently there are 400 cages for more than 4000 birds. Well-known pheasant species in the pheasantry comprise species inhabitant to Pakistan that are Koklass (*Pucrasia macrolopha)*, Kalij (*Lophura leucomelana)*, Monal *(Lophophorus impeganus)*, Cheer (*Catreus wallichi)* and Tragopan (*Tragopan melanocephalus*) and non-native species including the Golden Pheasant (*Chrysolophus pictus)*, Lady Amherst (*Chrysolophus amherstiae)*, Yellow Golden (*Chrysolophus pictus mut. luteus)* and Reeves pheasant *(Syrmaticus reevesii)* (Zaman, 2008). Genetic diversity has been calculated using many different types of data, which include qualitative and quantitative characters, chromosomes, nuclear DNA loci, chloroplast DNA, proteins and mitochondrial DNA. DNA based markers, provide powerful tools for calculating genetic relatedness between and within animals population. A variety of types of DNA markers have been used which include RFLP, RAPD, Minisatellite and Microsatellite DNA (Sharma *et al.*, 2001).

MATERIALS AND METHODS

DNA isolation and PCR formulation

Feathers of three pheasants *i.e.* lady amherst

* Corresponding author: drinamullah34@gmail.com
0030-9923/2015/0006-1739 $ 8.00/0

(*Chrysolophus amherstiae),* golden pheasant (*Chrysolophus pictus)* and yellow golden (*Chrysolophus pictus mut. luteus)* were collected from Dhodial Pheasantry, Mansehra and kept at -20°C till further processing. DNA extraction was carried out by a modified method adopted from Bello *et al.* (2001), include the following steps; The feathers were cut into small pieces of 0.5 to 1.0 cm, were ground using pestle and mortar and placed in a 1.5 ml Eppendorf tubes containing 700 µl of lysis buffer (50 mMTris-HCl pH 8, 20mM ethylenediaminetetra acetic acid, EDTA, pH 8, 2% sodium dodecyl sulfate SDS, and proteinase K at a final concentration of 75 µg/100 µl). The samples were incubated at 60°C for 16 h, inverting the tubes vigorously after every 45 min at room temperature. After lysis, the samples were vigorously vortexed for 15 seconds to homogenize the lysate and centrifuged at 10,000x g for 10 min at room temperature. The supernatant was transferred to a clean 1.5 ml Eppendorf tube, 400 µl phenols were added at room temperature and mixed by inverting the tubes. Samples were allowed to settle for 5 min and centrifuged at 10,000xg for 10 min at room temperature. Supernatant was transferred to fresh 1.5 ml Eppendorf tube, equal volume of isopropanol, 40 µl sodium acetate was added and mixed by inverting the tubes. Tubes were incubated at -20°C for 30 min and centrifuged at 10,000x g for 15 min. DNA pellet was washed with 70% ethanol and centrifuged at 10,000xg for 5 min at room temperature; pellet was air-dried and dissolved in 30 µl of double distilled water.

During the current study, isolated DNA was verified on agarose gel. Five µl DNA from each sample was mixed with 3 µl loading dye (Bromophenol blue 0.0092 ml, glycerol 0.23ml, 5xTBE 0.30ml and distilled water 1ml) and loaded in the wells. Gel was then run at a constant voltage at 70 volts for one hour. Components of PCR were the genomic DNA used as template, dNTPs (dATP, dCTP, dGTP and dTTP), RAPD primers (Table II) (Yeo *et al.*, 2000), Taq DNA polymerase buffer, $MgCl_2$ and Taq DNA polymerase. For statistical analysis, every band was considered as single locus/allele. Alleles/loci were scored as present (1) or absent (0). Bivariate 1-0 data matrix was generated and genetic distances (GD) among the genotypes were estimated using Unweighted Pair Group of Arithmetic Means (UPGMA) as described by Nei and Li (1979). The formula used to calculate GD is given below;

$$GD=1-d_{xy}/d_x+d_y-d_{xy}$$

GD, genetic distance; dx, unique band in genotype 1; Dy, unique band in genotype2; dxy, common band in genotype 1 and 2.

Table I.- Average estimates of genetic distances among 9 samples using fourteen RAPD primers.

	A	B	C	D	E	F	G	H
B	0.25							
C	0.21	0.19						
D	0.15	0.32	0.13					
E	0.14	0.26	0.29	0.14				
F	0.2	0.35	0.07	0.21	0.17			
G	0.50	0.48	0.47	0.43	0.43	0.43		
H	0.56	0.63	0.49	0.57	0.55	0.53	0.32	
I	0.46	0.60	0.45	0.51	0.47	0.52	0.12	0.43

Table II. - RAPD primers used during present study.

S.No	Primer ID or Oligo name	Primer sequences (5'–3')	%GC
1	GL.A-12	TCGGCGATAG	60
2	GL.A-15	TTCCGAACCC	60
3	GL.A-18	AGGTGACCGT	60
4	GL.B-11	GTAGHCCCGT	60
5	GL.B-12	CCTTGACGCA	60
6	GL.B-14	TCCGCTCTGG	70
7	GL.B-15	GGAGGGTGTT	60
8	GL.B-18	CCACAGCAGT	60
9	GL.B-20	GGACCCTTAC	60
10	GL.C-14	TGCGTGCTTG	60
11	GL.C-15	GACGGATCAG	60
12	GL.C-16	CACACTCCAG	60
13	GL.C-19	GTTGCCAGCC	70
14	GL.C-20	ACTTCGCCAC	60

RESULTS

Figures 1 and 2 showed bands patterns of alleles of different molecular weight ranging from 400bp–1000bp (estimated using molecular weight marker "100 bp gene ruler" Fermentas #SM0323) obtained from Gene Link, Inc, USA). A total of 40 alleles in 9 different samples were amplified giving an average of 4.4 alleles per sample. Estimates of genetic distances from 9 sample of pheasants ranged between 0– 63%. Maximum genetic distance (GD =

63%) was estimated for 6 comparison, 16 comparison showed complete homozygosity, while 15 comparisons showed 50% genetic distance.

Average GD among the 9 different samples estimated using the fourteen RPAD primers ranged from 7%–63% (Table I). Maximum average GD (63%) was estimated for one sample *viz.*, lady amherst sample B and yellow golden sample H. Minimum average GD (7%) was estimated for 1 sample. Rest of the samples showed varying range of average GDs from 8%-60%.

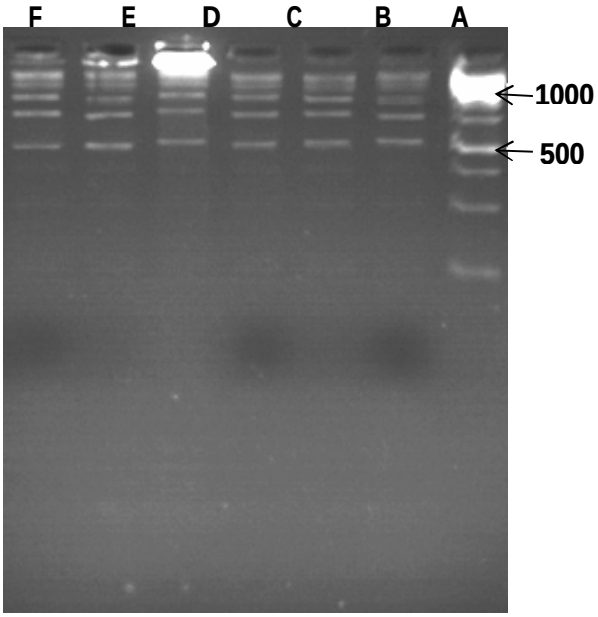

Fig. 1. PCR amplification profiles using RAPD primers GLA 12 and GLB 15. Lanes A-C: Lady Amherst (*Chrysolophus amherstiae).* Lanes D-F: golden pheasant (*Chrysolophus pictus).* Lane M: DNA marker (Fermentas #SM0323).

The resulting data of fourteen RAPD were used to analyze phylogenetic relationship among the samples. The 9 samples of pheasants were clustered in 2 groups A and B comprising 6 and 3 samples in each. Group A comprised lady amherst and golden pheasant, while group B comprised yellow golden samples.

DISCUSSION

The current study was carried to characterize nine samples of exotic pheasants through RAPD

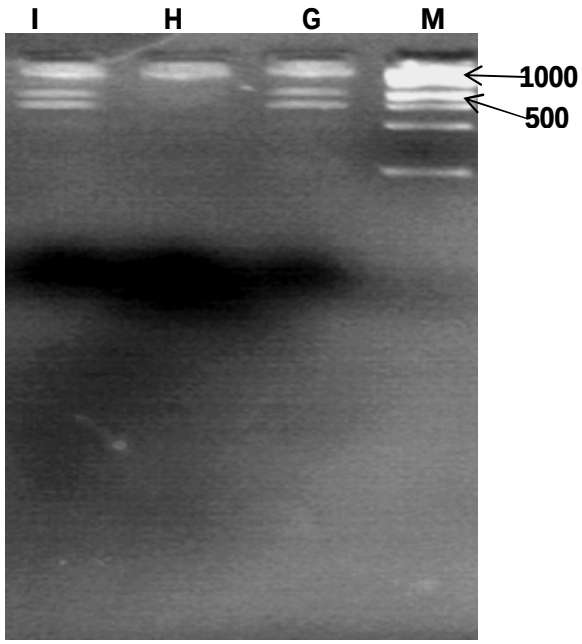

Fig. 2. PCR amplification profiles using RAPD Primer GLA 12 and GLB 15. Lanes G-I: Yellow Golden (*Chrysolophus pictus mut. luteus).* Lane M: DNA marker (Fermentas #SM0323).

analysis. For DNA extraction the protocol of Bello *et al. (*2001) was modified. The modifications included cutting of feathers into small pieces of 0.5 to 1.0 cm instead of grinding and incubation of the samples at 60°C for 16 h and shaking the tubes vigorously after every 45 min.

The PCR results were analyzed using computer package Popgene 32. For genetic diversity, fourteen RAPD primers were used (Yeo *et al.*, 2000). Nine samples of pheasants A, B, C, D, E, F, G, H and I were grouped into two clusters as A and B. Cluster A consisted of six samples from lady amherst and golden pheasant, while cluster B comprised of three samples of yellow golden.

Maximum GD (63%) was estimated for lady amherest and yellow golden. Minimum average GD (7%) was estimated yellow golden. Rest of the samples showed varying range of average genetic distances from 7-63%. It is evident from cluster analysis that lady amherst and yellow golden pheasant were most distantly related to each other. Some earlier researchers had reported 58–99% and

14–43% genetic distance in wild and captive individuals of Elliot pheasants (*Syrmaticus ellioti*) (Ping *et al*., 2005).

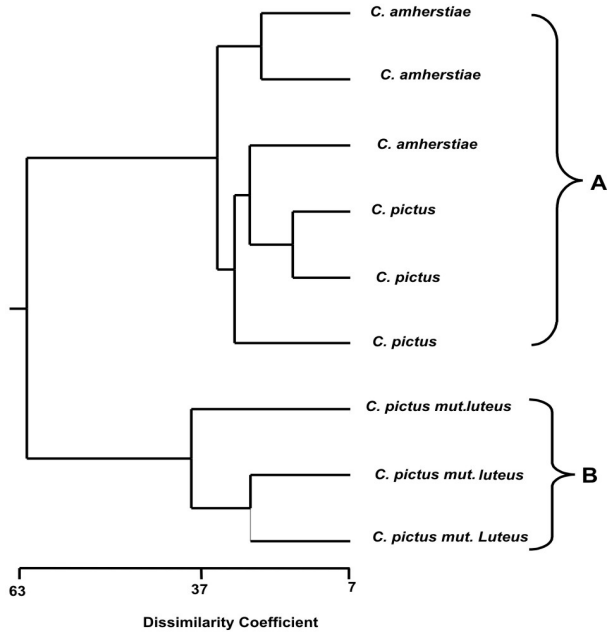

Fig. 3. Dendrogram showing phylogenetic relationship among 9 pheasant samples.

Vapa *et al*. (2007) had reported 14-43% genetic distance in *Phasianus* spp. Mehmood *et al.* (2009) had reported 4%–57% GD in *Lophophorus, Lophura, Catreus, Tragopan* and *Pucrasia* spp. In pheasants, one has to keep in mind that morphological changes (*e.g*., feathers, body weight, size and color) are not the result of adaptations to the environment, but have a social significance and thus may not be indicative of the genetic relationships. PCR based markers have proved to be very useful for the determination of genetic relationships among populations (Maurer *et al.*, 2005).

More molecular work is required to achieve a better understanding about diversity of pheasant genome. Extra nuclear DNA technologies should be explored for further understanding of pheasant genome and classification of this important bird. It is also recommended that in future breeding programs may be undertaken. Lady amherst and yellow golden pheasant be crossed to create maximum genetic variability in pheasants.

ACKNOWLEDGMENT

Wildlife Department and Department of Genetics, Hazara University, Mansehra are acknowledged for sample collection and provision of lab facilities, respectively.

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(Received 5 July 2014, revised 20 May 2015)