SSR Based Analyses of the Genetic Diversity of Pheasant Species of Pakistan

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ABSTRACT

Six pheasant species; koklass pheasant (*Pucrasia macrolopha*), Himalayan monal (*Lophophorus impejanus*), white crested kalij (*Lophura leucomelana*), western tragopan (*Tragopan melanocephalus*), cheer pheasant (*Catreus willachii*) and blue peacock (*Pavo cristatus*) found in Pakistan were analyzed for elaborating genetic diversity and their possible phylogenetic affinities. The total genomic DNA was isolated from blood and PCR was performed by using six SSR markers. The results revealed low to medium level genetic diversity (GD = 4–57%) among the pheasant's genotypes. Maximum GD (57%) was estimated between female of Himalayan monal and male of blue peacock while minimum GD (4%) was observed in two comparisons; one between male of monal and female of cheer pheasant and other between the male of western tragopan and female of cheer pheasant. Phylogeny was analyzed by constructing the dendrogram, based on PCR amplification profiles. Dendrogram clustered all the genotypes of pheasants into two main groups A and B. Group A was largest and subdivided into sub-groups. Some novel findings were also observed in dendrogram in which male and female of the same species were found to be clustered with male or female of other species in separate groups. This novelty needs further genetic elaborations for its clarity.

Key words: Genetic diversity, pheasants of Pakistan, Phylogenetic analysis, Microsatellites, SSR

1. Introduction

Pheasants belong to family Phasianidae in order Galliformes and are the most beautiful species of birds in the world for their brightly coloured plumages. They have small with large crypts dark to bright colored bodies and blunt-wings plumage with strong legs. Mostly, pheasants have elaborated head and neck ornamentation including feathers and wattles [1].Some species are sexually mono- or dimorphic [2].Galliformes is a large and diverse order consisting of approximately 70 genera and more than 250 Species [2, 3]. Phasianidae is generally comprised of three subfamilies; Tragopaninae, Argusianinae and much diverse Phasianinae which has 10 genera [4]. In Pakistan, six pheasant species; koklass pheasant (*Pucrasia macrolopha* Lesson, 1829), Himalayan monal (*Lophophorus impejanus* Latham, 1790), white crested kalij (*Lophura leucomelanos* Latham, 1790), western tragopan (*Tragopan melanocephalus* Gray, 1829), cheer pheasant (*Catreus willachii* Hardwicke, 1827) and blue peacock (*Pavo cristatus* Linnaeus, 1758) are reported [5]. Pheasants are precious and beautiful birds which are economically important to human beings. Feathers of some species are used for ornamentation and cloths manufacturing. Some species are important to the ecotourism industry. Some species have been domesticated and are reared for meat and eggs [6]. Pheasants are prominent figures in conservation campaigns and their colorful plumages and charismatic breeding behaviour have made them cultural icons [7, 8]. The present study was aimed to analyze the genotypes of pheasants of Pakistan for the estimation of genetic diversity (GD) and phylogenetic analysis. The genotypes were characterized by simple sequences repeats **(**SSR) markers. The SSR are short tandem repeats (STR) of genomic sequences and can be used according to the level of variation, which ranges from very low to extremely high [9].

2. Materials and Methods

Blood samples of pheasants were collected from Dhodial Pheasantry, Mansehra, Pakistan. Blood was taken from the ulnar superficial vein of the wing from healthy adults (male or female: 1: 1) by using hypodermic needle (27 gauge) and 5 ml disposables syringe. It was then immediately transferred into a 3

ml EDTA (ethylene diamine tetra acetate) tubes and stored at –20 ºC until further analysis. Total genomic DNA from the blood was extracted by following Weining and Langridge [10] with some modifications. First of all 300 µL blood sample was taken in an Eppendorf tube. Then 300 µL of DNA extraction buffer (SDS: 20%; Tris-Cl: 100 Mm; Nacl: 400 Mm; EDTA: 10 Mm; pH: 8.5) and 300 µL of phenol, chloroform and isoamyalcohol mixture (25: 24: 1) were added to the tube. The tubes were then homogenized in ice for 1 hour and centrifuged at 12000 rpm for 5 minutes. The aqueous phase was transferred to fresh tubes. In next step 30 µL of Sodium acetate (3 M; pH: 4.8) and 300 µL of isopropanol was added to the tube and mixed gently until the precipitate of DNA was visible. The tubes were then centrifuged at 12000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol, dried and dissolved in 40 µL TE buffer. The quality and quantity of DNA was checked on 1% agarose/TBE gel. Simple Sequence Repeats (SSR) primers (Table 1) were used for PCR. The components of PCR were DNA template, dNtPs (dAtP, dCtP, dGtP and dTtP), SSR Primer [11], $MgCl_2$ and Taq DNA polymerase. Denaturation, annealing and extension were carried out at 94, 52 and 72 ºC, respectively up to 40 cycles and PCR products were separated on 2% Agarose/TBE gel.

2.1 Statistical analysis

Data were analyzed using unweighted pair of group arithmetic mean (UPGAM) [12] and dendrogram was constructed using computer program POPGENE Ver. 3.2 [13].

3. Results

Out of ten SSR primers tested only six (Cp1, Cp2, Cp3, Cp4 Cp7, Cp12) amplified the genotypes of pheasants (Figure 1). All the PCR profiles were observed for banding patterns. Alleles were scored as present (1) or absent (0) and bivariate (1-0) data matrices were generated for individual primers amplification profiles. A total of 13 alleles were amplified by SSR primer set Cp12 (Figure 1-a) giving an average of

1.2 allele in eleven genotypes of pheasants. Total genomic DNA of one genotype (cheer pheasant male) was not amplified by this set of primer and hence its result is not included in analysis. The alleles ranged 250-300 bp in size. Estimates of genetic distances ranged from 0-100%. Twelve comparisons showed maximum genetic distance (100%) while 31 comparisons revealed no difference (GD = 0%) among the genotypes.

Table 1. List of SSR primer sets used in present study.

S. No.	Primer ID	Sequences (5'–3')	Anneal. Temp. °C
1	CP1	F: TCAGTGGAAGGGTTCATGACT	52
		R:GCTACTTTCCTGGTGGCCTA	
2	CP2	F:AAGGACAATGTAATTGGTGCTACA	52
		R:TCACAGAGCCATCAGGAATG	
3	CP3	F: CATTGCTGCTAGGCGAATTT	50
		R: TGACGTCATGAGCAACACAA	
4	CP4	F:GACCACCTAGAGCAGGTTGC	52
		R: TGGGCACTGACTGAAAGACA	
5	CP7	F: TGCCCATGACATTTAACCAA	50
		R: TTGGGCT GCTTAGGCATGTAAGG	
6	CP12	F:CCGCTGGAGTTCACCTTTAC	52
		R:CATGCTGACGATGGAGAAGA	

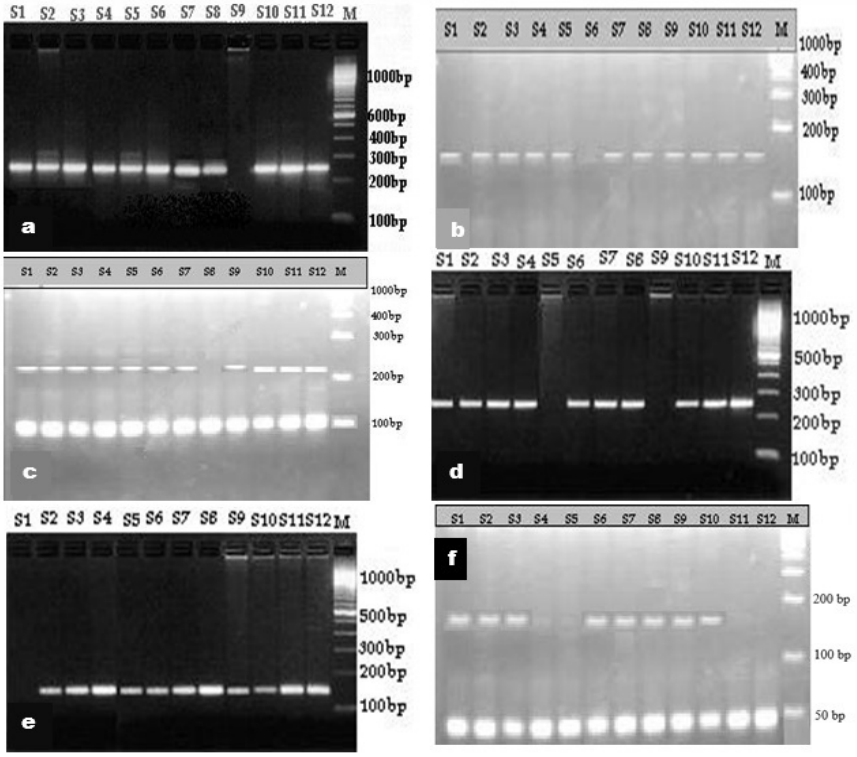

Figure 1. Polymerase chain reaction (PCR) amplification profiles of 12 genotypes of pheasants (S1 & S2:koklass male and female; S3&S4: Himalayan monal male and female; S5&S6: white crested kalij male and female; S7&S8: western tragopan male and female; S9&S10: cheer pheasant male and female; S11&S12: blue peacock male and female respectively) by using 6 SSR primers sets: Cp12 (a); Cp1 (b); Cp3 (c); Cp7 (d); Cp2 (e); Cp4 (f).

The primer set Cp1 showed little amplification (Figure 1-b). The total genomic DNA of one genotype (white crested Kalij female) was not amplified by Cp1 so its result was excluded from analysis. In rest of the 11 genotypes of pheasants a total of 11 alleles were amplified by primer set Cp1 at an average of 1 allele per genotype. The amplicons ranged in size approximately 150 bp. The estimates of genetic distances ranged 0-100%. The minimum genetic distance 0% was observed in 26 comparisons while 19 comparisons showed maximum genetic distance (100%) among the twelve genotypes of pheasants.

Table 2. Average estimates of genetic distances among 12 genotypes of pheasants (S1: koklass male; S2: koklass female; S3: Himalayan monal male; S4: Himalayan monal female; S5: white crested kalij male; S6: white crested kalij female; S7: western tragopan male; S8: western tragopan female; S9: cheer pheasant male; S10: cheer pheasant female; S11: blue peacock male; S12: blue peacock female) by using 6 SSR primers sets during present study.

	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
S1	0.30	0.28	0.38	0.43	0.30	0.18	0.33	0.46	0.31	0.35	0.35
S2		0.27	0.29	0.38	0.29	0.20	0.28	0.38	0.24	0.22	0.28
S3			0.28	0.43	0.39	0.09	0.13	0.36	0.04	0.22	0.22
S4				0.36	0.39	0.27	0.35	0.46	0.31	0.57	0.32
S5					0.40	0.46	0.54	0.36	0.50	0.51	0.51
S6						0.42	0.50	0.31	0.38	0.41	0.41
S7							0.09	0.36	0.04	0.27	0.27
S8								0.50	0.09	0.36	0.36
S9									0.46	0.43	0.52
S10										0.28	0.28
S11											0.09

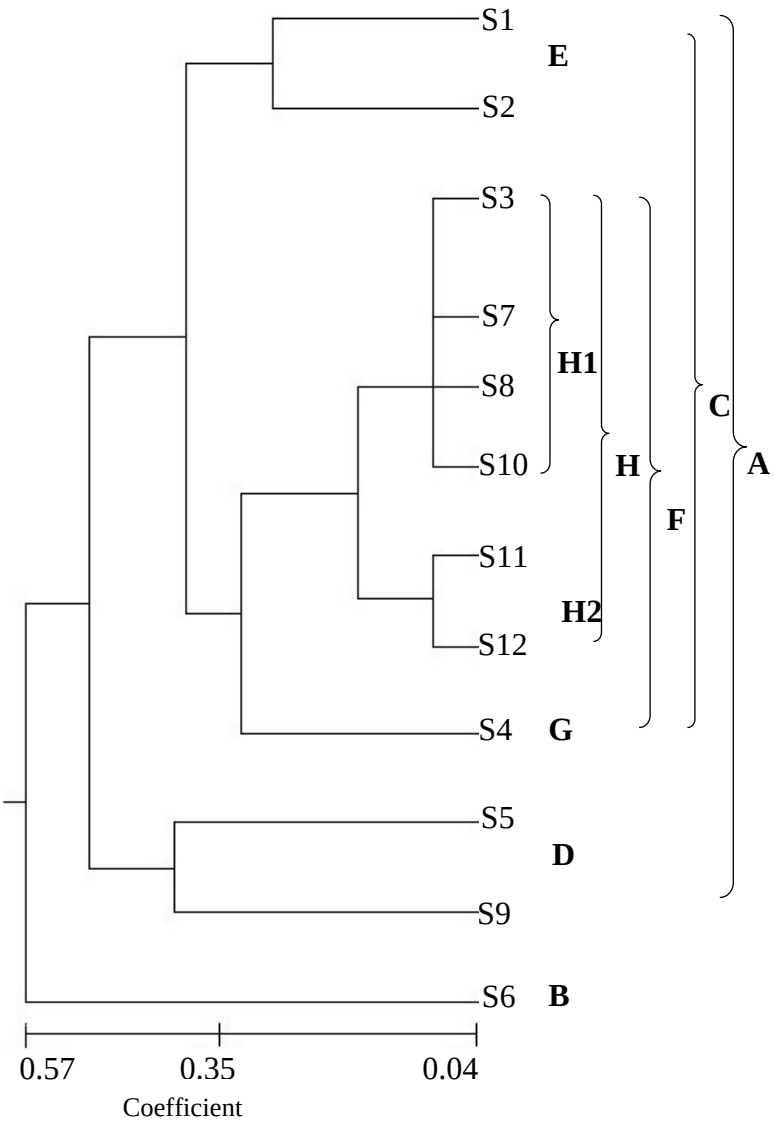

Figure 2. Dendrogram constructed for 12 genotypes of pheasants (S1: koklass male; S2: koklass female; S3: Himalayan monal male; S4: Himalayan monal female; S5: white crested kalij male; S6: white crested kalij female; S7: western tragopan male; S8: western tragopan female; S9: cheer pheasant male; S10: cheer pheasant female; S11: blue peacock male; S12: blue peacock female) based on data obtained by using 6 SSR primers sets during present study.

The PCR-amplification profile of primer set Cp3 (Figure 1-c) showed various levels of genetic polymorphism among the genotypes of pheasants. A total of 23

alleles were amplified at an average of 1.9 alleles per genotype. The amplified fragments ranged in size from 100-220 bp. Range of genetic distances estimated for primer set Cp3 was 0-100%. Maximum genetic distance was observed 100% in 11 comparisons while 26 comparisons were found completely homozygous for the amplified loci.

A total of 10 alleles were observed in PCR-amplification profile of primer set Cp7 (Figure 1-d). Two genotypes (white crested Kalij male and cheer pheasant male) were not amplified by this set of primer so were excluded from analysis. The amplified alleles ranged 250 bp in size. Range of genetic distances remained 0-100%. Twenty compressions were 100% heterozygous while 46 were observed 100% homozygous.

The PCR-amplification profile of primer set Cp2 (Figure 1-e) showed a total of 11 alleles in eleven genotypes and one genotype (Koklass male) was not amplified by this set of primer so it was not included in analysis. The amplified fragments ranged 150 bp in size. Estimates for genetic distance ranged 0-100%. Minimum genetic distance (0%) was observed in 55 comparisons while 11 comparisons showed maximum genetic distance (100%) among the genotypes of pheasants.

The primer set Cp4 amplified a total of 20 alleles in all the genotypes, giving an average of 1.7 alleles per genotype (Figure 1-f). The size of amplicons ranged 50-180 bp. The estimated genetic distance ranged 0-100%. The minimum GD was calculated 0% for 28 comparisons while maximum was 100% in 38 comparisons.

Average genetic distances of 12 genotypes of pheasants by six SSR primers ranged 4-57% (Table 2). Maximum average GD (57%) was observed in one comparison (Himalayan monal female-blue peacock male), closely followed (54%) by (white crested Kalij male-western tragopan female) while minimum average GD (4%) was noted for two comparisons (Himalayan monal male-cheer pheasant female and western tragopan male-cheer pheasant female) closely followed (9%) by four comparisons (Himalayan monal male-western tragopan male; western tragopan male- western tragopan female; western tragopan female- cheer pheasant female; blue peacock male- blue peacock female).

The bivariate data was also used to construct a dendrogram (Figure 2) for phylogenetic analysis by using computer program POPGENE Ver. 3.2. All the genotypes of pheasants were clustered into two main groups A and B. The group A was largest and consisted on 11 genotypes. The group B consisted on only one genotype. The group A was sub divided into C, D, E, F, G and H sub groups. Some novel findings were also observed in dendrogram which clustered the female of white crested Kalij into a separate group B while the males of white crested Kalij and cheer pheasant were placed together in sub-group D. Similarly female of cheer pheasant was clustered into subgroup H1 along with male of Himalayan Monal and genotypes of male and female of Western Tragopan. The female of Himalayan Monal was put separately into sub-group G.

4. Discussion

Estimation of genetic diversity is a prerequisite for improving any species or genetic stock. Traditionally, classifications of pheasants were based on phenotypic traits. In some cases, recent genetic studies have found differences in the structure proposed [14]. Various procedures (morphological, cytological and biochemical markers) have been utilized in the past for the estimation of genetic diversity in various plants and animal species of commercial importance. These markers were not considered suitable for large scale utilization mainly because of their limited numbers and difficult, expensive and time consuming assay procedures [15, 16]. With the recent introduction of DNA technology, marker assisted selection (MAS) of suitable genotypes has been utilized extensively. These DNA based markers include PCR based assay, restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) etc. Among PCR based assay, various primer systems viz: allele specific amplification (ASA), cleavage amplification polymorphism sequence (CAPS), sequence tag site (SAS) etc have been used [17]. In present study we used the SSR markers which are spread throughout the eukaryotic genome. These repeats are highly polymorphic even among closely related species due to the mutations, causing variation in the number of repeating units. The SSR assay

is being increasingly used due to its relative advantages; are highly polymorphic and thus highly informative, moreover, they can be analyzed rapidly and technically simple PCR-based assay, further, they are co-dominant markers and finally SSRs are abundant and uniformly distributed. The present study is the first documented report on DNA based characterization of pheasants in Pakistan. As pheasants are sensitive to habitat degradation, vulnerable to human exploitation and have a central position in food web so they are valuable bio-indicators [18]. Jiang et al. [19] reported 0.93-1% genetic similarity among wild populations of Elliot pheasants (*Syrmaticus ellioti*). Vapa et al. [14] observed 14-43% genetic distance in *Phasianus spp*. Yinhua et al. [20] tested the micro satellite markers and observed 4-88% genetic distances among the ducks, peacock, chicken and goose.

The twelve pheasants genotypes studied during present research provided interesting results. It is evident from the average genetic distance estimates that low-medium amount of genetic variability (GD ranging from 4–57%) was present among the pheasants genotypes. Cluster analysis showed expected results for four species i.e, koklass, Himalayan monal, western tragopan and the blue peacock, whose males and females were clustered together into the same sub-groups whereas novelty was observed in case of kalij and cheer pheasants, whose males and females were placed into separate groups and sub-groups. This finding was further strengthened by genetic distance estimates (Table 2). High GD (46%) was estimated between male and female of cheer pheasants and the similar case was for kalij pheasant's male and female (GD 40%). The findings indicate the potentially differences in genomic structures of the two sexes of the same species. This novelty needs further genetic elaborations of both the sexes for its clarity.

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