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RAPD based Genetic Diversity of Endangered Himalayan Gray Langur (*Semnopithecus ajax*) Populations of Pakistan

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ABSTRACT

Gray langurs (*Semnopithecus* spp.) are leaf eating monkeys fragmentally distributed in northern hills of Khyber Pakhtunkhwa (KP) and Azad Jammu and Kashmir (AJK). It was believed that different fragmented populations of these monkeys might have experienced a genetic isolation or inbreeding. We assessed the level of genetic diversity and genetic isolation of different langur populations using Randomly Amplified Polymorphic DNA (RAPD) genetic markers. We collected 86 different samples (feces 64, hair 13, blood 5, tissues 4) from 5 geographic langur populations of Pakistan and Azad Jammu and Kashmir (AJK) and succeeded in extraction of DNA from 23 samples, which were used for further genetic analysis. RAPD makers (n=8) produced 245 bands (30.62±2.87 Mean±SE / primer) of different molecular weights (126-3342 bp), of which, 96 were population specific. Polymorphism was (37.71±5.29%; mean ± SE), with the highest in Muzaffarabad population (54.29%), followed by Poonch (43.67%) and Neelum (36.73%). Values of Shannon's (I: 0.129-0.200) and Nei's genetic diversity (He: 0.082-0.117) indices were low. Total heterozygosity (Ht: 0.144±0.007), genetic diversity within population (Hs: 0.096±0.005), between populations (Dst: 0.018±0.003), genetic differentiation constants among populations (Gst: 0.153±0.025) and within populations (Rst: 0.847±0.025) were calculated. Gene flow (Nm: 3.246±0.448) and genetic similarity (97-98%) between populations was high. UPGMA based dendrogram identified five distinct geographic groups, and Mantel tests (*Rxy*=-0.008, P>0.05) suggested a non-significant relationship between genetic distance and geographic distance. Phist (Φ_{PT}) value suggested a significance difference in within population and between populations ($_{PT}$=0.042; p=0.006) variances, suggesting that within populations variation was higher (96%) than variation between populations (4%). Present study suggested a low level of genetic diversity; however, gene flow was higher suggesting low chances of inbreeding between gray langur populations of Pakistan and AJK.

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Authors' Contribution
RAM designed the study, collected field data, extracted genome and wrote the article. MH and SSB amplified the primers. BA and MSA statistically analyzed the data. MNK and AM supervised the study and revised the manuscript.

Key words
Genetic diversity, Himalayan gray langur, RAPD, Pakistan.

INTRODUCTION

Kashmir gray langur or Himalayan gray langur (*Semnopithecus ajax*) is an endangered species of old-world monkey fragmentally distributed in different areas of northern Pakistan (Khyber Pukhtunkhwa (KP) and Azad Jammu and Kashmir (AJK)) (Roberts, 1997; Minhas *et al*., 2012). In AJK, these monkeys have been recorded in District Neelum, Muzaffarabad, Jhelum valley and Bagh (Ahmed *et al*., 1999; Minhas *et al*., 2012). In KP, they have been reported from different localities of district Mansehra and Kohistan (Roberts, 1997; Minhas *et al*., 2012). There are geographical and ecological reasons to believe that different populations of langur were isolated from one another, but the level of isolation was not known. Threatened species with isolated populations of few breeding individuals often have lower genetic diversity (Lacy, 1997). Knowledge of the extent of isolations existing between different populations and sizes of individual populations has a conservation value. Smaller isolated populations having higher chances of being genetically fixed through inbreeding and dwindle to extinction due to accumulation of unflavored alleles and genetic homogeneity (Lacy, 1997). Use of molecular genetic markers in assessment of the conservation status of a population/species is a new trend. Genetic variability in the population has a predictive value for the potentials of the species/population to cope with future environmental fluctuations and its continued survival under environmental odds (Frankham *et al*., 2002). Information of genetic variation within and among populations and applications of genetic principles are considered as crucial scientific tools for wildlife managers in making proper decisions for conserving populations (Bellemain, 2004).

The assessment of genetic diversity has been made easier with the development of molecular genetic markers.

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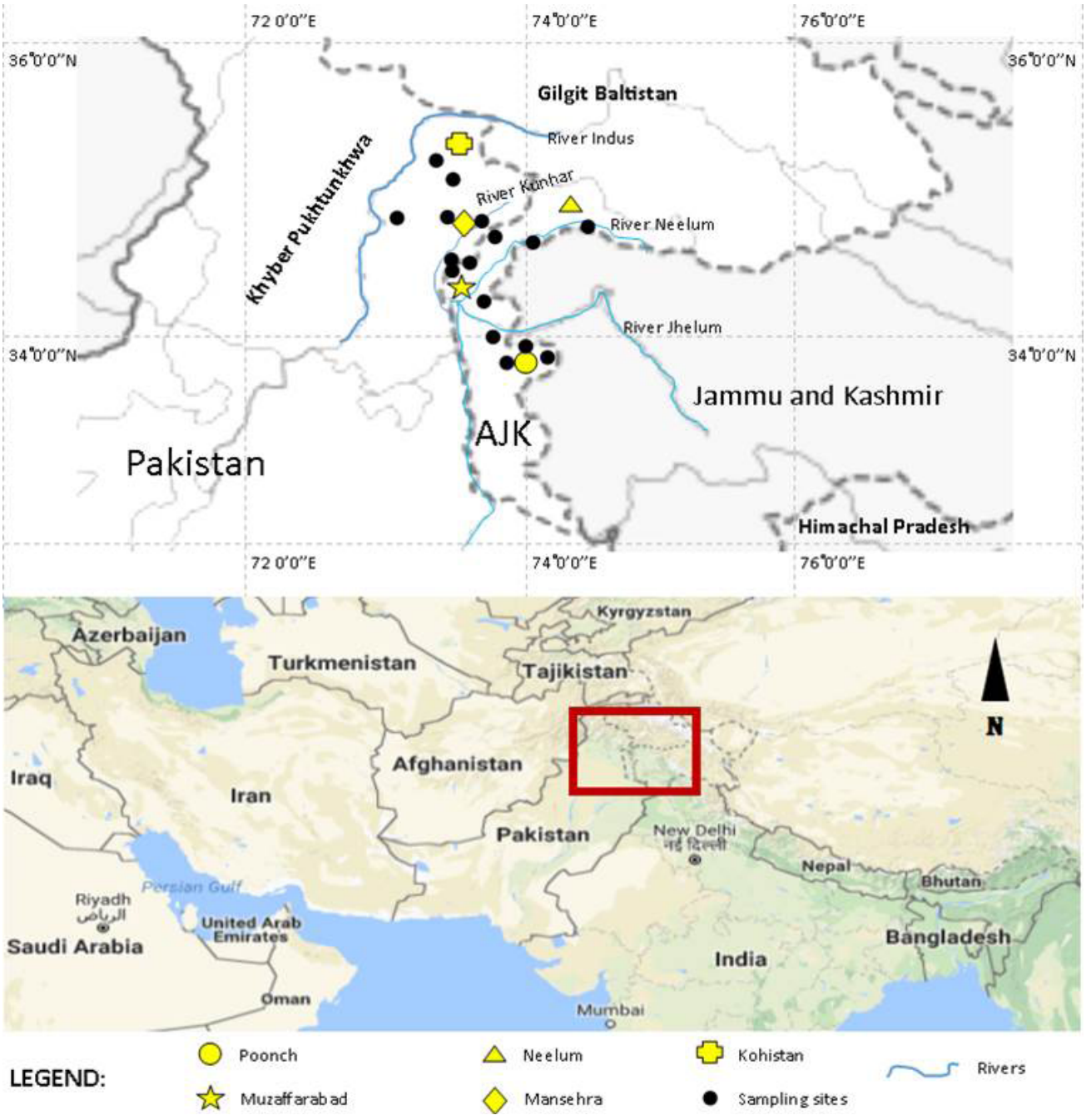

Fig. 1. Location map of gray langur populations with sampling localities in Pakistan.

Molecular genetic markers are heritable characters with multiple states ranging between one, two or multiple states (alleles) per character (locus). All genetic markers reflect variations in DNA sequences, usually with a trade-off between precision and convenience (Sunnucks, 2000). Several molecular markers have been used in genetic diversity studies, including, informative or co-dominant markers, e.g., microsatellite (SSRs) and restriction fragment length polymorphism (RFLP), and non-informative or dominant markers, such as, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP; Nassiry *et al.*, 2009). RAPD markers are easily developed and analyze using PCR amplification techniques. During RAPD

analysis the banding patterns of two closely related species/populations are more similar than the evolutionary unrelated species/populations (Miesfeld, 1999). RAPD technique has become popular for genetic comparison of organisms where relatively small-scale DNA sequences are compared using many genetic markers without cloning and sequencing of the genome of the species in question (Kumar and Gurusubramanian, 2011). These markers are used for genetic diversity analysis of different vertebrates due to limitation in cost, time and labour (Kumar and Gurusubramanian, 2011; Vasave *et al*., 2014; Shafi *et al*., 2016; Mudasir *et al*., 2016). These markers have also been used for genetic studies in different taxa of primates including langurs (Ding *et al*., 2000; Arif and Khan, 2009).

The study was based upon the hypothesis that different inbreeding populations of the gray langur distributed in Pakistan and Azad Jammu and Kashmir are isolated to different degrees from one another. Gray langur, being endangered in Pakistan/AJK, we planned to identify individual populations and characterize them using molecular genetic tools to work out the intra-specific variability using molecular genetic analysis. These research findings will have a value for the conservation biologists and managers in settling the present status of langurs in Pakistan and can be used in development of future langur population management strategy.

MATERIALS AND METHODS

Sampling

Based on information about langur population, available with literature and wildlife staff, a reconnaissance survey of potential gray langur areas was conducted in 2012-2013. A total of 86 samples (feces 64, hair 13, blood 5, tissues 4) were collected from 5 localities *viz*. Palas valley, district Kohistan, KP (34°52'-35°16' N, 72°52'-73°35' E); Allai valley, District Battagram, KP (34°33'-34°47' N, 72°54' -73°15' E); Kaghan valley, District Mansehra, KP (34°14′-35°11′ N, 72°49′-74°08′ E); Machiara National Park, AJK (34º-31' N, 73º-37' E); Neelum valley, AJK (34°28′-34°48′ N, 73°91'-74°58′ E); Jhelum valley, AJK (34°23'44.4"-34°08'29.7" N, 73°31'44.2"-73°56'12.6" E); Las Danna, AJK (33.9212° N, 73.9550° E) and Haveli Kahuta, AJK (33.8841°N, 74.1083° E) (Fig. 1).

For collection of fresh fecal material, langur troops were followed and about 20-50 gm of out layers of fecal samples were collected in sterilized polypropylene tubes containing 95% ethanol following Nsubuga *et al*. (2004) and Zhang *et al*. (2006). Hair samples, collected from the roosting sites/resting rocks/bushes were removed with forceps and transferred to a clean paper envelope and packed separately in zipped polyethylene bags and stored at 4ºC till DNA was extracted and analyzed. Tissues samples (skin, muscle, liver) were collected from the carcasses of leopard/human killed (n=4) and were preserved in sterilized polyethylene bags polypropylene tubes filled with 95% ethanol or simply collected in sterile polyethylene bags. Dried muscle samples (n=3) were also collected from preserved material, available locally, and stored in clean sterile paper envelopes, and stored at -20°C. Blood (n=5) samples were collected from langurs freshly killed (n = 2; within 24 hours) or maintained in illegal custody (n=3), using 10 ml sterile syringes (BD, USA), transferred to 10 ml potassium EDTA vacutainer tubes (BD, USA), labeled, and stored at 4°C, in refrigerator in the laboratory.

Ethics statement

This study did not require approval by any ethics committee as all samples were collected through noninvasive methods without incision of live wild animals. However, for field samplings a permit was acquired by the Department of Wildlife vide letter Number. WL&F/713/2015, dated November 16, 2015. The blood samples were collected with the help of a trained veterinarian through the sterile syringes from individuals illegally held by the locals.

DNA extraction

DNA was extracted from blood tissues by using method of Sambrook *et al*. (1989); however, for DNA extraction from fresh and dried tissues, some modifications were carried out. To modify this method, tissues were washed twice with 100% ethanol (by dipping for 15 min) and distilled water (10 min) each and grinded using liquid nitrogen. To the powdered tissues, 750 µl of Solution A (0.32 M Sucrose; 10 mM Tris-pH 7.5; 5 mM $MgCl_2$; 1% (v/v) Triton X-100) was added and incubated overnight at 37°C. On the next day after centrifugation for 1 min at 13000 rpm, the supernatant was discarded and the nuclear pellet mixed with 500 µl of Solution B (10 mM Tris-pH 7.5; 400 mM NaCl, 2 mM EDTA - pH 8.0) along with 20 µl of 20% SDS and proteinase-K each. This mixture was incubated at 37°C for 24-36 h. For rest of the steps, protocol of Sambrook *et al*. (1989) was followed. From hair, follicles of about one cm from the root were cut by sterile razor blade and washed with 95% ethanol and then distilled water. Subsequent steps were the same as described above for blood/tissues. The extracted DNA was dissolved in 50-70 µl of TE buffer. Methods of Zhang *et al*. (2006) was adopted for extraction DNA from fecal samples. While extraction DNA from fecal materials, negative extraction controls were also processed following Liu *et al*. (2008). For confirmation of DNA presence, the

extracted DNA was run on 1% agarose gel and visualized using gel documentation system (Vilber Lourmat Bio-Print-ST4, France) for evaluation the quality, while quantity of DNA was assessed using spectrophotometer (UV3000, Germany). DNA samples with 1.7-2.0 absorbance ratios were subjected to PCR amplification.

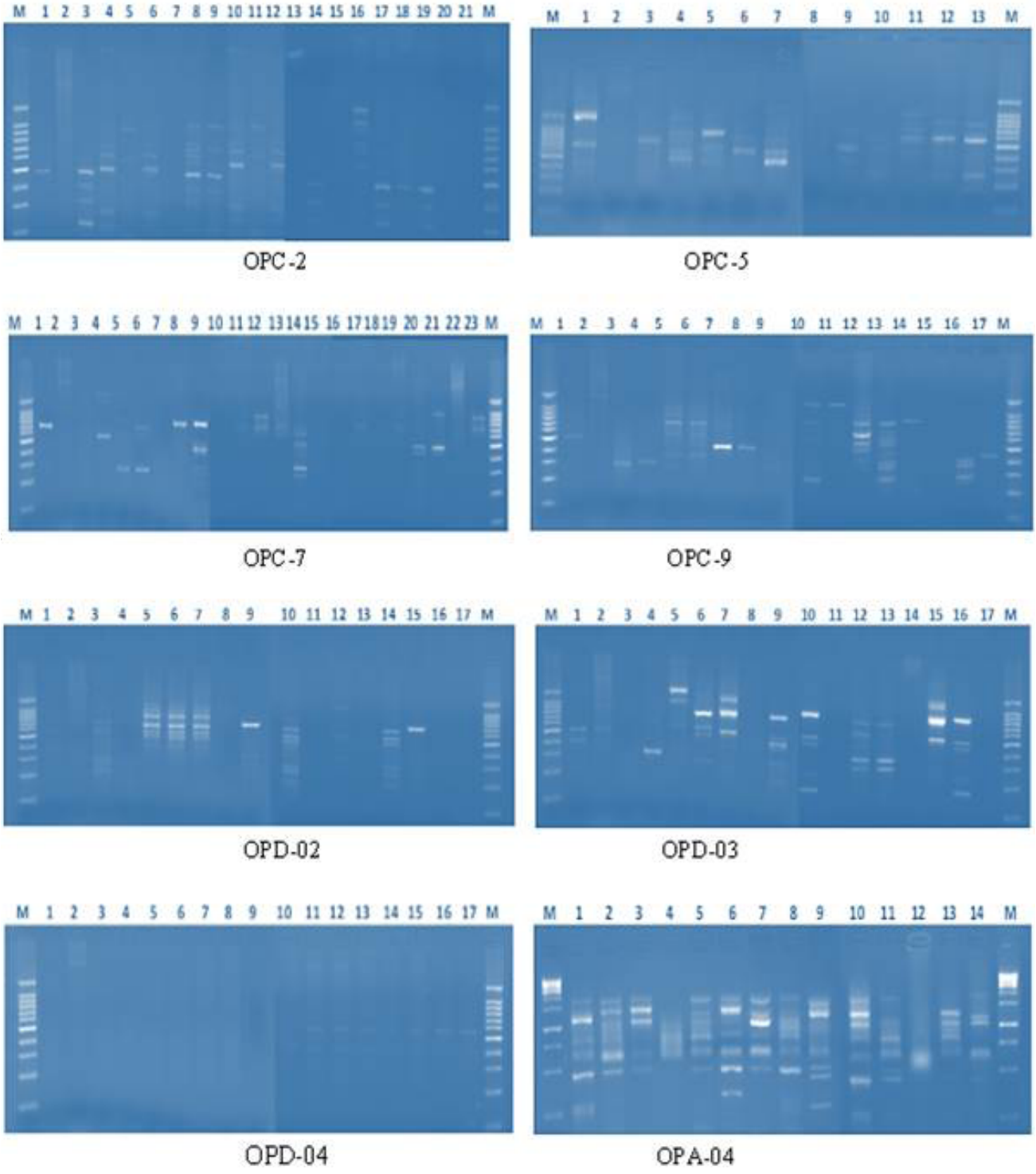

Fig. 2. Gel pictures (2%) of PCR products of RAPD markers (1-8) for gray langur populations of Pakistan (M=100bp/1kb Ladder).

PCR optimization and amplification

Eight primers were used for amplification of 23 DNA samples of gray langur (Table I) and these primers were randomly selected from published papers (Ding *et al*., 2000; Shafi *et al*., 2016). The PCR reaction consisted of initial denaturation at 94ºC for 5 min followed by 39 cycles of 1 min denaturation at 94ºC, annealing at different temperatures (specific to each primer) for 30 sec to 1 min and extension of strand at 72ºC for 1 min. To ensure the completion of extension process, a further extension at 72ºC was carried out for 10 min. For optimization of the annealing temperatures of primers were adjusted by lowering the 2-5 degrees from the calculated melting temperatures by using the equation T_m = 4(G+C) + 2(A+T), where 'G', 'C', 'A', and 'T' represent the number of respective nucleotides in the primer. The amplification success and quality of the PCR products of RAPD was assessed by performing 2% agarose gel electrophoresis. Gel documentation system was used for visualization of the quality of the amplified products and picturizing of the band patterns. The gel photographs of the amplified products were used for further analysis by scoring the absence or presence of different bands (Fig. 2).

Table I.- RAPD markers used in genotyping of Himalayan gray langurs (after Ding *et al*., 2000).

Primer	Sequence	G+C content (%)
OPC-2	5'-GTGAGGCGTC-3'	70
OPC-5	5'-GATGACCGCC-3'	70
OPC-7	5'-GTCCCGACGA-3'	70
OPC-9	5'-CTCACCGTCC-3'	70
OPD-02	5'-GGACCCAACC-3'	70
OPD-03	5'-GTCGCCGTCA-3'	70
OPD-04	5'-TCTGGTGAGG-3'	60
OPA-04	5'-AATCGGGCTG-3'	60

Discriminatory powers of markers

To assess the discriminatory powers of markers, each RAPD marker was analyzed for different informative indices, including polymorphism information content (PIC), resolving power (RP), marker index (MI), and effective multiplex ratio (EMR), calculated in MS Excel 2016 using the following relationships (Mandal *et al*., 2016; Pecina-Quintero *et al*., 2012; Kayis *et al*., 2010): PIC = $2f_i$ $(1-f_i)$; where, f_i is the frequency of marker band present and $1-f_i$ is the frequency of marker absent bands (Roldàn-Ruiz *et al*., 2000) MI = PIC × number of polymorphic loci, or, MI = PIC × EMR, where, EMR = nβ is the effective multiplex ratio, measured as the product of the total number of loci per fragments per primer (n) and the fraction of polymorphic loci fragments (β) (Pecina-Quintero *et al*., 2012). RP = Σ Ib, were "Ib" is the informativeness of band, calculated as Ib=1-[2 × (0.5-p)], being "p" the proportion of each genotype containing the band. The diversity index (DI), indicating the genetic diversity or expected heterozygosity at the locus, = 1 - 1/L ΣPi2, where, Pi is the allele frequency in (each individual allele is considered a unique fragment amplification) and L is the number of loci (Mandal *et al*., 2016).

Genetic data analysis

Scoring of bands: Beside visual observations, the Software (GelAnalyzer-2010a) was used to record presence or absence of bands (Arruda *et al*., 2012; Abeykoon *et al*., 2015). Binary matrix of each genotype was recognized as presence "1" and absence "0" of bands. The percentage of polymorphic bands and bivariate (1-0) data matrix for each primer was analyzed using the software (MS Excel 2016, GenAlEx-6.5; Popgene-1.31). Using presence or absence data, the calculations of genetic distances (Fitzpatrick, 2009) and the degrees of similarities or dissimilarities of RAPD fragments between individuals and populations were carried out using Popgene and GenAlEx-6.5 (Peakall and Smouse, 2012).

Genetic diversity, genetic differentiation and gene flow

For calculation of different genetic diversity attributes including allele frequencies, total numbers of observed (Na) and effective alleles (Ne), genetic diversities, Shannon' Information Index (I), Nei's Gene Diversity Index (He) or expected heterozygosity, total heterozygosity (Ht), genetic diversity within (Hs) and between (Dst) populations, genetic differentiation within (Rst) and between (Gst) populations and gene flow (N_m) were calculated and analyzed by using the Popgene (1.32), GenAlEx (6.5) and MS Excel (2016) software programs (Yeh *et al*., 1999; Gaudeul *et al*., 2004).

Genetic similarity and genetic distance

Nei's genetic similarities and distances were calculated based on pairwise population matrix of different populations using GenAlEx (6.5). Cluster analysis was carried out by constructing dendrograms in Popgene (1.32) program using un-weighed pair group mathematical averages (UPGMA). These dendrograms were based on Nei's genetic distances/similarities data for different populations as well as different genotypes (Nei, 1972, 1978). Clustering of different genotypes from gray langur populations was performed using Euclidean similarity index based on Paired group cluster analysis of binary data (present/absent) produced by different markers in Past (3.16) software (Hammer *et al*., 2001).

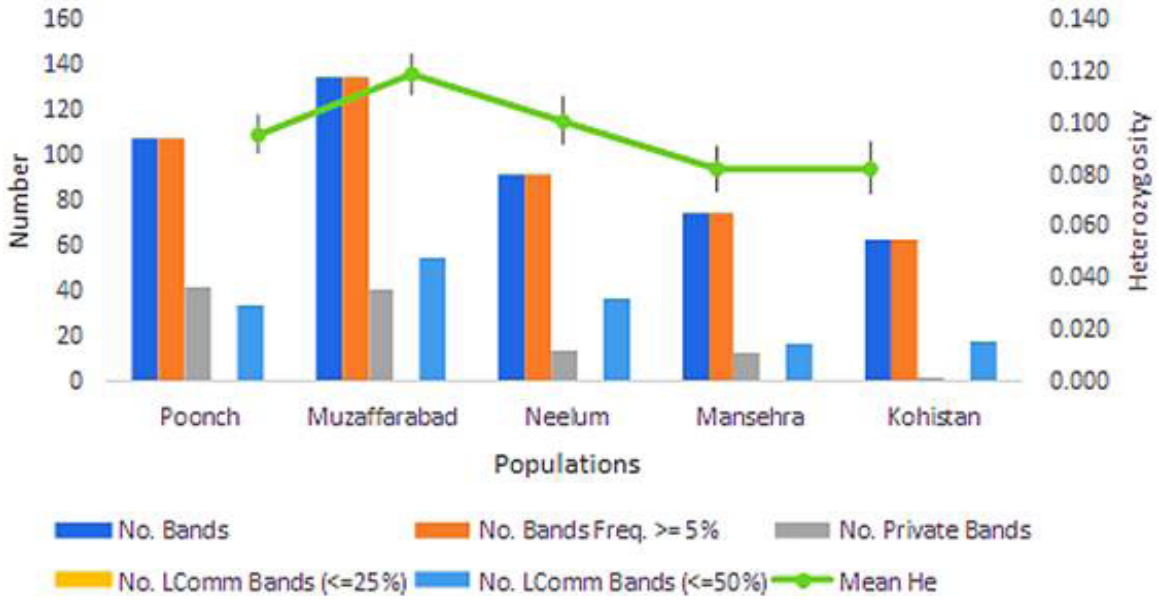

Fig. 3. Total Band Patterns for RAPD markers across different populations of gray langur in Pakistan. No. Bands, number of different bands; No. Private Bands, number of bands unique to a single population; He, expected heterozygosity.

AMOVA and PCoA

The analysis of molecular variance (AMOVA) was carried out to calculate the *F*-statistics and/or their analogues for estimation of hierarchical partitioning of genetic variation, variance components and their significance level within populations and among populations or regions (Fitzpatrick, 2009). The AMOVA analysis was done using GenAlEx 6.5 software program (Gaudeul *et al*., 2004). Principal Coordinate Analysis (PCoA) or Classical Multidimensional Scaling was carried out using GenAlEx 6.5. PCoA calculated from geographic distance of different genotypes was calculated based on Global Position System coordinates.

Spatial genetic analysis

To analyze the distribution patterns of genetic variations across different spatial scales, Spatial Genetic Analysis was also done (Diniz-Filho *et al*., 2013). Global Position System technology was used to collect latitude-longitude coordinates used for regression analyses in conjunction with genetic distances by using Mantel tests (Mantel, 1967) in GenAlEx 6.5 software.

RESULTS

DNA extraction

Extraction of DNA was tried from 86 different field samples using standardized methods. However, the successful extractions and amplification was possible for 23 samples with the overall success rate of 24.41%.

Table II.- Discriminatory powers analysis for different RAPD markers used in genetic analysis of gray langurs of Pakistan.

Markers	MI	EMR	RP	PIC
OPC-2	6.63	39.00	7.62	0.17
OPC-5	8.68	28.00	6.19	0.31
OPC-7	10.91	37.00	7.24	0.29
OPC-9	10.20	30.00	6.57	0.34
OPD-02	12.16	38.00	8.10	0.32
OPD-03	10.88	32.00	7.52	0.34
OPD-04	4.90	14.00	3.05	0.35
OPA-04	9.17	27.00	10.00	0.34
Total	73.54	245.00	56.29	2.46
Mean	9.19	30.63	7.04	0.31
SE	2.417	2.878	0.699	0.021

MI, marker index; EMR, effective multiplex ratio; RP, resolving power; PIC, polymorphism information content.

Discriminatory power of markers

Values of discriminatory power indices of different markers have been presented in Table II. The mean PIC value (±SE) was 0.31±0.02, which indicated that all markers used were highly polymorphic and efficient.

The primers with higher RP values are considered more informative in distinguishing the genotypes (Heikrujam *et al*., 2015).

Allelic diversity

The highest numbers of total bands and number of different bands with a frequency of >5% was recorded in Muzaffarabad population (n=134) and lowest in Kohistan population (n=62) (Fig. 3).

Observed and effective alleles

Muzaffarabad population has highest mean numbers of observed alleles 1.077± 0.063 and effective alleles 1.158±0.013 at different loci and Kohistan population has lowest mean numbers of observed alleles 0.511±0.043 and effective alleles 1.134±0.013 (Table III). On average, the primer OPA-04 revealed the maximum numbers of observed (0.889±0.098) and effective (1.200) alleles, while OPC-7 exhibited the lowest numbers of observed (0.638±0.075) and effective alleles (1.113±0.008; Table III).

Polymorphism

Amplification of RAPD loci exhibited different patterns based on relative frequency (%) of polymorphism in different gray langur populations (Fig. 4). The highest frequency of polymorphic loci was recorded in Muzaffarabad population (54.29%) and lowest was recorded in Kohistan population (25.5%), while the mean polymorphism was 37.71±5.29 (%±SE).

Shannon's information and Nei's heterozygosity indices

Mean (±SE) values of Shannon's index (I) 0.200±0.013 and heterozygosity or Nei's genetic diversity index (He) 0.117±0.008 were recorded highest in Muzaffarabad population and lowest in Kohistan population *i.e.* (I) 0.129±0.010 and (He) 0.084±0.007, respectively (Table IV). During current study, the mean values of the Shannon's index and Nei's genetic diversity index in all populations was in the ranges of 0.129-0.200 and 0.082-0.117, respectively (Table IV).

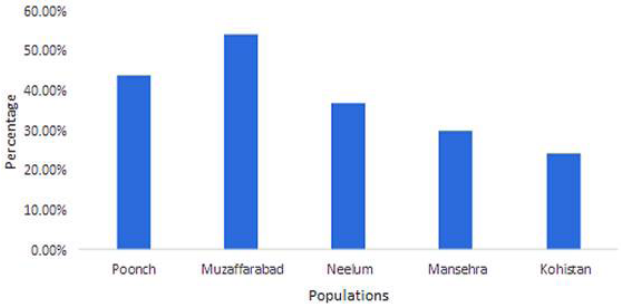

Fig. 4. Frequency of polymorphic loci (%) in different populations of gray langur in Pakistan.

Table III.- Summary of allelic diversity exhibited by RAPD markers in gray langur populations in Pakistan.

Populations		OPC-2	OPC-5	OPC-7	OPC-9	OPD-02	OPD-03	OPD-04	OPA-04	Mean	SE
Poonch	BF	0.077	0.093	0.092	0.087	0.105	0.125	0.086	0.170	0.104	0.011
	NA	0.769	0.786	0.811	0.800	0.895	1.063	0.857	1.037	0.877	0.040
	NE	1.090	1.113	1.111	1.103	1.128	1.152	1.100	1.216	1.127	0.014
Muzaffarabad	BF	0.144	0.114	0.086	0.147	0.126	0.150	0.100	0.207	0.134	0.013
	NA	1.231	1.071	0.757	1.267	1.158	1.125	0.857	1.148	1.077	0.063
	NE	1.174	1.136	1.105	1.179	1.152	1.183	1.121	1.214	1.158	0.013
Neelum	BF	0.077	0.107	0.108	0.142	0.125	0.109	0.125	0.176	0.121	0.010
	NA	0.564	0.714	0.649	1.000	0.789	0.688	0.571	0.926	0.738	0.056
	NE	1.096	1.137	1.139	1.178	1.159	1.142	1.165	1.179	1.149	0.010
Mansehra	BF	0.103	0.107	0.074	0.100	0.066	0.078	0.071	0.194	0.099	0.015
	NA	0.769	0.714	0.595	0.600	0.421	0.500	0.429	0.704	0.591	0.047
	NE	1.127	1.137	1.090	1.131	1.085	1.101	1.094	1.204	1.121	0.014
Kohistan	BF	0.077	0.143	0.090	0.056	0.105	0.115	0.190	0.173	0.119	0.017
	NA	0.410	0.607	0.378	0.333	0.526	0.563	0.643	0.630	0.511	0.043
	NE	1.101	1.141	1.124	1.071	1.140	1.153	1.160	1.185	1.134	0.013

BF, band frequency; Na, number of alleles; Ne, number of effective alleles.

Table IV.- Summary of different genetic diversity constants exhibited by RAPD markers in gray langur populations in Pakistan.

Populations		OPC-2	OPC-5	OPC-7	OPC-9	OPD-02	OPD-03	OPD-04	OPA-04	Mean	SE
Poonch	I	0.130	0.146	0.147	0.141	0.166	0.198	0.144	0.231	0.163	0.012
	He	0.073	0.086	0.085	0.081	0.097	0.115	0.081	0.145	0.095	0.008
Muzaffarabad	I	0.228	0.188	0.138	0.231	0.204	0.219	0.159	0.234	0.200	0.013
	He	0.133	0.107	0.08	0.134	0.117	0.131	0.092	0.144	0.117	0.008
Neelum	I	0.116	0.156	0.147	0.211	0.174	0.155	0.149	0.198	0.163	0.011
	He	0.070	0.096	0.092	0.128	0.108	0.097	0.098	0.123	0.102	0.006
Mansehra	I	0.157	0.156	0.117	0.139	0.094	0.112	0.099	0.176	0.131	0.011
	He	0.094	0.096	0.069	0.088	0.058	0.069	0.063	0.117	0.082	0.007
Kohistan	I	0.103	0.143	0.107	0.079	0.136	0.147	0.151	0.164	0.129	0.010
	He	0.066	0.092	0.072	0.05	0.089	0.096	0.099	0.110	0.084	0.007
Mean	I	0.147	0.158	0.131	0.160	0.155	0.166	0.140	0.201	0.157	0.007
	He	0.087	0.095	0.080	0.096	0.094	0.102	0.087	0.128	0.096	0.007
SE	I	0.022	0.008	0.008	0.027	0.019	0.019	0.011	0.014		
	He	0.012	0.003	0.004	0.016	0.010	0.010	0.007	0.007		

I, Shannon's Information Index = -1* (p * Ln (p) + q * Ln(q)); He, Expected Heterozygosity = 2 * p * q; where for Diploid Binary data and assuming Hardy-Weinberg Equilibrium, q = (1 - Band Freq.) ^0.5 and p = 1 - q.

Table V.- Total heterozygosity, genetic diversity, genetic differentiation and gene flow between different populations of gray langur as exhibited by RAPD markers.

Markers	Total heterozygosity (Ht)	Genetic diversity		Genetic differentiation		Gene flow (Nm)
		Hs	Dst	Gst	Rst	
OPC-2	0.096	0.087	0.009	0.094	0.906	4.809
OPC-5	0.118	0.096	0.021	0.182	0.818	2.253
OPC-7	0.093	0.080	0.013	0.145	0.855	2.958
OPC-9	0.106	0.096	0.010	0.095	0.905	4.748
OPD-02	0.106	0.094	0.012	0.111	0.889	3.989
OPD-03	0.116	0.102	0.015	0.126	0.874	3.460
OPD-04	0.125	0.087	0.038	0.307	0.693	1.127
OPA-04	0.152	0.128	0.024	0.160	0.840	2.621
Mean	0.114	0.096	0.018	0.153	0.847	3.246
SE	0.007	0.005	0.003	0.025	0.025	0.448

Hs, genetic diversity within population; Dst, genetic diversity between population; Gst, genetic differentiation among population; Rst, genetic differentiation within population.

Total heterozygosity or heterogeneity (Ht)

The mean (±SE) value of total heterozygosity or heterogeneity (Ht) as exhibited by different RAPD primers among all populations of gray langurs was 0.144±0.007 (Table V). The highest value of Ht was expressed by the OPA-04 (0.152) followed by OPD-04 (0.125) and OPC-5 (0.118). The lowest value of Ht was showed by the OPC-7 (0.093).

Genetic diversity within and between populations

The mean value of genetic diversity (Hs) within population as exhibited by all RAPD markers for all populations was 0.096±0.005, while the mean genetic diversity between populations (Dst) was calculated as 0.018±0.003 (Table V). This indicated very low genetic variations within populations than the variation between different populations.

Gene flow

The mean values (±SE) of genetic differentiation constants among populations (Gst) and within populations (Rst) were 0.153±0.025 and 0.847±0.025. These low values of Gst and higher values of Rst are indicative of a higher gene flow between populations, and thus a lower level of genetic isolation between populations. Therefore, the mean value of gene flow/numbers of migrants (Nm) between populations was higher (3.246±0.448) ranging between 4.809 (OPC-2) and 1.127 (OPD-04; Table V).

The gene flow (Nm) was significantly correlated with genetic diversity between population (Dst; r= -0.919, $p<0.01$), differentiation among populations (Gst; r= -0.926, $p<0.001$) and within populations (Rst; r=0.926, $p<0.001$; Table VI). Genetic diversity between population (Dst) was also strongly correlated with genetic differentiation within populations (Rst; r= -0.968, $p<0.0001$) and among populations (Gst; r=0.968, $p<0.0001$). Similarly, and total heterozygosity between population (Ht) was strongly correlated with heterozygosity within population (Hs; r= 0.856, $p<0.01$).

Table VI.- Correlation matrix between total heterozygosity (Ht), genetic diversity within (Hs) and between (Dst) populations, genetic differentiation within (Rst) and between (Gst) populations as exhibited by RAPD markers in gray langur.

	Ht	Hs	Dst	Gst	Rst	Nm
Ht	1.000					
Hs	0.856	1.000				
Dst	0.648	0.160	1.000			
Gst	0.442	-0.085	0.968	1.000		
Rst	-0.442	0.085	-0.968	-1.000	1.000	
Nm	-0.542	-0.077	-0.919	-0.926	0.926	1

Table VII.- Pairwise population matrix of Nei genetic identity (above diagonal) and genetic distance (below diagonal) exhibited by RAPD markers in gray langur populations of Pakistan.

Populations	PCH	M/Abad	NLM	MNS	KTN
PCH	***	0.984	0.981	0.982	0.973
M/Abad	0.016	***	0.983	0.980	0.971
NLM	0.019	0.018	***	0.981	0.972
MNS	0.018	0.020	0.019	***	0.976
KTN	0.028	0.030	0.029	0.024	***

PCH, Poonch; M/Abad, Muzaffarabad; NLM, Neelum; MNS, Mansehra; KTN, Kohistan.

Genetic similarity and genetic distance

Nei's genetic similarities and distances were calculated using pairwise population matrix of different populations. As expected, the values of genetic similarities were very high (97-98%) among populations of gray langurs found in Pakistan/AJK. Inversely, values of genetic distances were very low (< 3%) between different populations (Table VII). Higher values of genetic similarity and lower values of genetic distance between different populations indicate a higher rate of gene flow and lower heterogeneity (Table V).

Based on genetic distance, dendrogram constructed using UPGMA in Popgene (1.32) divided the whole population of gray langurs into five groups (Fig. 5). Poonch and Muzaffarabad populations very closely related to each other forming a clade, while all others are comparatively distinctly related to each other. Neelum population is out-group of the Muzaffarabad-Poonch clade. Kohistan populations form separate clades. Muzaffarabad and Kohistan populations were the least related (genetic distance 0.030; Table VII).

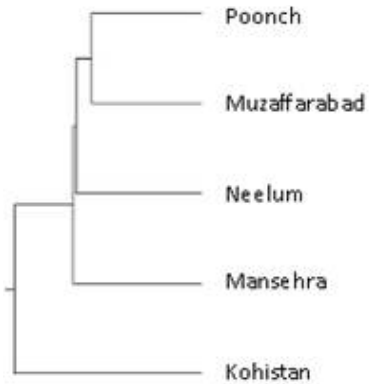

Fig. 5. UPMGA dendrogram based on Nei's genetic distance exhibited by RAPD markers in different populations of gray langurs.

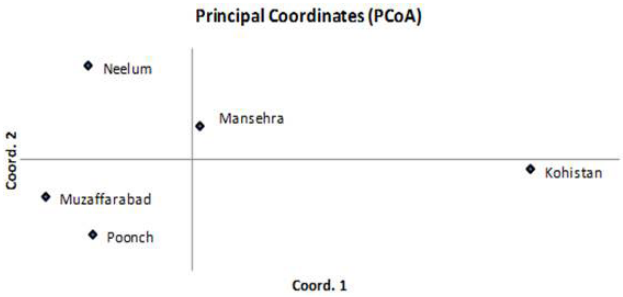

Fig. 6. Graphical presentation of PCoA results for different populations of gray langur.

Multivariate analysis

Principal coordinate analysis (PCoA) is a spatial way of ordinating the data to visualize the patterns of genetic relationship contained in larger matrices which are impossible to read and interpret. Three-dimensional principal coordinate analysis accounted for 38.99%, 22.29% and 21.21% of the variability as explained by the first three axes with a total 82.50% variability. Based on the maximum variability among first two axes of the Eigen values, the PCoA output has produced three or four distinct clusters. Two populations of AJK (Muzaffarabad-Poonch) form one cluster, while, Neelum, Mansehra and Kohistan populations formed three separate clusters (Fig. 6). PCoA analysis also provided similar clustering pattern as already suggested by dendrograms. PCoA calculated from genetic distance and similarity matrices of genotypes exhibited a lower (25.56%) percentage of variability than the different populations (82.50%). Based on first two axes of Eigen values, the PCoA results showed different patterns of genotypes (Fig. 7). PCoA calculated from geographic distance of different genotypes obtained by using the global position system coordinates in GenAlEX

6.5 exhibited a higher (81.91%) percentage of variability as also exhibited by the genetic distance (82.50%). Based on first two axes of Eigen values, the PCoA results showed three main clusters of genotypes (Fig. 8). These clusters are coinciding with the PCoA results based on genetic distance.

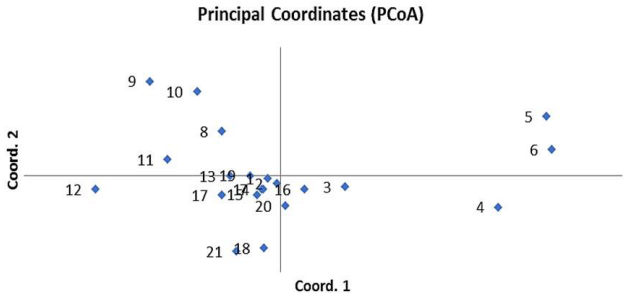

Fig. 7. Graphical presentation of PCoA results for different genotypes of gray langur.

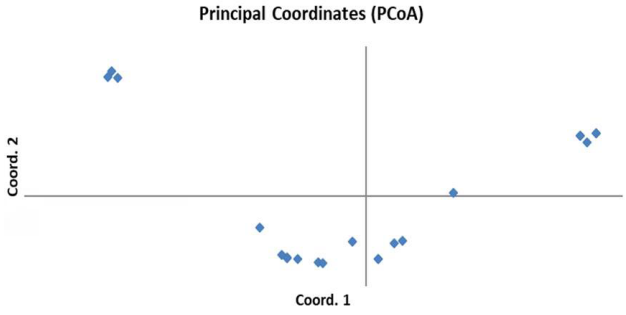

Fig. 8. Graphical presentation of PCoA results based on geographic distance for different genotypes of gray langur.

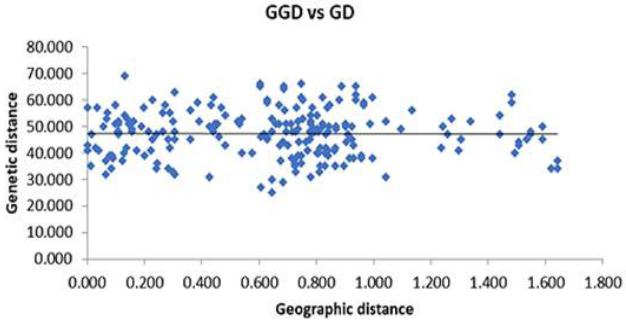

Fig. 9. Association between RAPD based genetic and geographic distances as depicted by Mantel Test.

Spatial genetic analysis

The calculated value of *Rxy*=-0.008, with P>0.05 revealed a nonsignificant relationship between genetic distance and geographic distance (Fig. 9). Hence, the genetic distances between different genotypes are not related to the geographic isolation considerably. Absence of any significant correlation between genetic and geographical distance suggests that geographic distance is not significantly working as effective restricting factor in gene flow between populations as already confirmed by the values of gene flow (Nm).

Analysis of molecular variance (AMOVA)

Analysis of molecular variance exhibited only 4% variance between populations and 96% variance within populations. The Phist (Φ_{PT}) value suggested a significance difference in the variance within population and between populations (Φ_{PT} =0.042; p=0.006). The results suggested that the variance contributed by individual variation within populations was higher (96%) than variation between population (4%).

DISCUSSION

Gray langurs are facing population decline throughout their distribution range including Pakistan (Groves and Molur, 2008; Nag *et al*., 2011; Minhas *et al*., 2012). From Himalayan species, *S. ajax* is considered endangered because of its restricted range distribution and continuous population decline (Groves and Molur, 2008). We used RAPD technology to assess the level of genetic isolation in different populations of gray langurs found in Pakistan. We used 8 different RAPD markers for this analysis. The average values of PIC and other parameters of these genetic markers assessment showed that, all genetic markers were highly effective and efficient markers. PIC values range from zero (monomorphic markers) to 0.5 for markers which is present in 50% of the individuals and absent in others 50% (Roldàn-Ruiz *et al*., 2000). The higher values of PIC suggest that, all markers used had the higher levels of efficiency and capability for genetic analysis in the current study. A remarkable difference in the banding patterns, percentages of polymorphic loci and higher PIC values in different langurs' populations indicated a high discriminatory potentials and usefulness of RAPD markers in genetic analysis.

The number and size of fragments/alleles produced correspond to the nucleotide sequence of the primer and DNA template, which is represented in the genome specific fingerprints of RAPD fragments (Welsh *et al*., 1991). Deferential amplification, as depicted in distinct band sizes, indicated a high genetic variability in different gray langur populations of Pakistan. The average values of observed (na), and effective number of alleles (ne) and percentage of polymorphic loci are considered as indicators of the actual level of genetic variability in the species under question. They high level of polymorphism and unique

patterns of bands also indicated the highly variability of different gray langur populations found in different areas, as also reported in different earlier studies (Ding *et al*., 2000; Sanches *et al*., 2012). The results also indicated that RAPD–PCR technique has potentials of revealing genetic variations among different populations. These techniques have been used in several studies in different vertebrate species including primates (Ding *et al*., 2000; Rodrigues *et al*., 2007; Kumar and Gurusubramanian, 2011; Vasave *et al*., 2014; Shafi *et al*., 2016; Mudasir *et al*., 2016).

A relatively larger number of unique bands have been recorded in different populations. The presences of these bands in different populations requires further investigation that, why do they exist? Is their existence a normal phenomenon? Did they arise due to population isolation or inbreeding?, as already highlighted by Schroeder *et al*. (2009). However, the negative values of inbreeding coefficient (*Fis)* in all populations suggested that, currently there is no significant inbreeding effect to any population and all populations exhibited heterozygosity excess.

The highest genetic distance was recorded between Muzaffarabad and Kohistan populations. Cluster analysis showed that Poonch and Muzaffarabad populations formed a monophyletic clade, while, all other populations formed almost separate groups. These affinities of populations are acceptable under the current geographic conditions. To detect the distribution patterns of genetic variations across different spatial scales, spatial genetic analysis was also done (Diniz-Filho *et al*., 2013). RAPD maker showed non-significant relationship between these distance (*Rxy*=-0.008, P>0.05) suggesting that genetic distance or isolation by distance was not related to the geographic distance (Mantel, 1967).

Higher values of genetic similarity and lower values of genetic distance between different populations indicate a higher rate of gene flow and lower heterogeneity. Seasonal movements of troops and individuals have been recorded in gray langur (Minhas *et al*., 2012). During these movements, inter-population gene flow possibly occurs. This allows us to propose that physical/habitat barriers expected for the area are not effective in genetically separating different langur populations, and all gray langur populations of Pakistan and AJK act as a single genetic unit. The present findings about different genetic diversity constants suggest high dissimilarities in genome compositions of different populations of gray langur found in Pakistan and AJK. These values of genetic diversity (I=0.129-0.200 and He=0.082-0.117) were lower as compared to those suggested by Ding *et al*. (2000). Using RAPD markers, they calculated the mean values of Shannon's index as 0.55 and 0.85 with the ranges between 0.00-0.162 and 0.27-1.51 in white headed langurs and black langurs, respectively. Similar values of genetic diversity indices (I=0.13 and He=0.20) have been recorded by Shafi *et al*. (2016) in a RAPD based study on *Tor putitora*. Rodrigues *et al*. (2007) calculated the mean values of Nei's diversity index as 0.26 in a RAPD based study on *Ozotoceros bezoarticus*. Based on the calculated values of the gene diversity indices and as compared with the above studies, it can be assessed that, a moderate level of genetic diversity is present in different langur populations found in Pakistan.

The mean value of gene flow (3.246) between all populations (>1) indicated that, more than one individuals migrate between different population to ensure the genetic flow. The values of Nm > 1 suggest a higher level of genetic flow existing between different populations (Mallet *et al*., 1990). Similar findings have (Nm=3.22) been reported by Shafi *et al*. (2016) for *Tor putitora* using RAPD markers. The finding of the present study also suggests that, the inter-population migration of individuals still exists. Therefore, the probability of inbreeding and genetic drift is low in these populations, indicating that, the genetic fixation is not a serious problem for the existing langur populations. Slatkin (1987) suggested that values of Nm >1 (*i.e*., above the minimum number of migrants per generation) needed to avoid differentiation by genetic drift. Population grouping developed by different dendrogram cluster based on genetic distances and similarities seems natural, as expected under the geographic conditions. It appears that despite the presence of possible physical/habitat barriers different geographic populations of gray langur are not completely isolated from one another and gene flow persists between these populations as indicated by the higher values of gene flow exhibited in present study. The lowest similarity between Poonch/Muzaffarabad and Kohistan populations can be expected on distance logic, being located on two extremes of the range Kohistan population is geographically placed at the wider distances from other population, hence, emerged as a separate cluster. However, it is also linked to other population via the adjacent Mansehra population.

CONCLUSIONS

Concludingly, this study suggested a low level of genetic diversity among different gray langur populations of Pakistan and AJK. Currently, the study did not find any risk of genetic isolation in these sub-populations. However, a higher number of private bands might be the indication that different populations may face the risk of genetic isolation or inbreeding in future. Thus, we suggest that further confirmatory studies should be conducted using other molecular techniques.

Statement of conflict of interest

Authors have declared no conflict of interest.

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