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# GENETIC DIVERSITY AMONG COMMON LEOPARDS INHABITING AZAD JAMMU AND KASHMIR, PAKISTAN

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## ABSTRACT

The common leopard (*Pantherapardus*) is one of the highly threatened species of big cats in Pakistan. Azad Jammu and Kashmir (AJK) is among the main distribution areas of these leopards but due to poaching, habitat fragmentation and other anthropogenic activities, there is a rapid decline of the population in the state. We carried out the study to analyze the genetic diversity of common leopard in Azad Jammu and Kashmir (AJK).We used skin samples that were collected from the killed leopards by farmers and local hunters from six different localities of AJK. We used phenol chloroform triton modified method to extract the DNA and used four SSR markers (FCA043, FCA026, FCA075 and FCA090) to analyze genetic diversity by using PCR-based simple sequence repeat (SSR) analysis. We also calculated Allelic frequencies, Nei's genetic diversities index(within and among populations) and Shannon information index with POPGENE 32 program which showed that highest genetic distance (0.4275) was established between the Kotli (Pop-6) and Hattian (Pop-3) populations, and lowest between Hattian (Pop-3) and Neelum (Pop-2) populations (0.0393) of common leopard. Mean gene flow (Nm) was found to be 0.8030 which showed low level of gene flow between different populations and higher level genetic differentiation between populations supported our hypothesis of habitat fragmentation.

**Keywords:** SSR analysis, gene flow, genetic distance.

## INTRODUCTION

The common leopard (*Pantherapardus*) is an adoptable big cat belongs to Felidaef amily (Nowell and Jackson,1996). The geographical range of these leopards spanned all of sub-Saharan and North Africa, the Middle East and Asia Minor, South and Southeast Asia, and extended to the Amur Valley in the Russian Far East (Seidensticker and Lumpkin, 1991; Nowell and Jackson, 1996). Common leopard is considered as near threatened on the IUCN Red list because its population is declining in large parts of its range due to habitat loss, fragmentation, and hunting for trade and pest control. The status assessment of mammals categorized the common leopard as a critically endangered species in Pakistan (Sheikh and Molur, 2005). In Pakistan, these leopards are found in the highlands of Baluchistan, Sindh and mountain forests of Punjab, Khyber-Pakhtoonkhawa and Azad Jammu and Kashmir (AJK). It is widely accepted that knowledge of phylogenetic history and genetic variation is of fundamental importance for threatened species conservation (Lande,1999). Many studies have been conducted by using different types of molecular markers for the estimation of different levels of genetic variation among species (Baker,1992; Castric *et al*., 2001).

According to Luikart *et al*. (1998) for conservation of endangered species the alteration in DNA sequence and microsatellite markers for the analysis of genetic variation has critical role. Microsatellites are powerful markers, extremely used for estimating genetic diversity, forensic studies, genome mapping, conservation and management of biological diversity and population genetics (Kim, 2003; Uphyrkina *et al*.2001 Hatanaka *et al*., 2006; Primmer *et al*., 2006). tried to assess the molecular genetics differentiation, variation in existing leopard populations and geographical division by using molecular methods and short tandem repeat loci (STRs) or 25 feline-specific microsatellites.Miththapala*et al*.(1996) resolved six phylogeographic groups of leopards by using three molecular genetics methods (allozymses, RFLP –mtDNA and minisatellites). Barnett *et al*., 2009; Eckert *et al*., 2010 and Wilting *et al*., 2011 analyzed the genetic variation in different animals' population and studied the phylogenetic history and hybridization rates.

Menotti-Raymond *et al*.,(1999) used 253 microsatellite loci in felines and developed a genetic linkage map (Mondol *et al*., 2009) reported that 25 specific microsatellite markers showed positive amplification in 37 wild caught leopard's samples. In Pakistan/AJK the genetic structure and taxonomic status of common leopard is still poorly understood. Hence the identification of taxonomic and populations units and understanding evolutionary relationships of leopard in Pakistan is essential for the conservation of their biological diversity (Allendorf and Luikart, 2007).

Therefore, we conducted the study for the genetic diversity of these leopards with the specific objectives to assess the genetic diversity among and within different populations, estimation of allelic frequencies and analysis of possible chances of genetic drift in different common leopard populations found in various localities of AJK.

## MATERIALS AND METHODS

**Sample Collection:** For DNA extraction, we acquired tissue samples (skin/hair; n=18, formalin preserved)of dead animals from the AJK Wildlife Department which were collected from the killed leopards (*Pantherapardus*) by local hunters and farmers from different localities of Azad Jammu and Kashmir(AJK). We stored these samples at -20°Cbefore using it for further processing. Based on the origin of these skins, we categorized these samples into six different populations of AJK; District Muzaffarabad (pop-1), District Neelum (pop-2), District Hattian (pop-3), District Poonch(pop-4), DistrictBhimber (pop-5) and District Kotli (pop-6). We carried out the molecular work at Molecular Ecology Laboratory ofBioresource Research Centre, Islamabad.

**DNA Extraction and Quantification:** We used the phenol chloroform triton modified method for DNA extraction. To modify phenol chloroform triton method, we washed the skin samples with $dH_2O$ and TE buffer separately before extracting DNA. Then we added 1ml of $dH_2O$ in samples and centrifuged the samples at 13000 rpm for 10 minutes and then we collected pellet. Afterwards,we added 100ml of TE buffer and centrifuged the samples at 13000rpm for 10 min and discarded supernatant. Then, we extracted DNA by using the Phenol-chloroform-Triton method; 300 µl lysis solutions (200mM NaCl, 10mM Tris-Hcl, pH 8.0, 10mM EDTA, pH 8.0 and 0.1% Triton-100) added along with about equal amount of 1M DTT (40µl) and 40µl Proteinase K (2 mg/ml). We followed the method of Sambrook *et al.* (1989) and precipitated the DNA with equal volume of isopropanol and 3M of sodium acetate. Then, we washed DNA with 70% ethanol and re-suspended in TE buffer (10 mM Tris HCl, pH 8.0, and 1 mMEDTA) and stored at -20$^o$C until use. We checked the extracted DNA on 1% agarose gel and assessed the quality and quantity of extracted DNA by spectrophotometric method.

**Polymerase Chain Reaction:** We used 4 polymorphic microsatellites loci, (Table 1)to carry out PCR amplification. We selected these markers from originally designed microsatellite for the domestic cat i.e.*Feliscatus* (Menotti-Raymond *et al.*, 1999). PCR amplification of the template DNA samples in a total volume of 25 µL was carried out and the optimized conditions are as follows: Total reaction mixture containing 50ng of DNA template, 1X of Taq buffer, 1.5 mM $MgCl_2$, 0.2mM of each dNTPs, 1.5 U/µL Taq DNA Polymerase (FermentasUAB, Lithuania) and 5pmol of each primer. We used PCR profile: Pre Denaturation at 94°C for 5 minutes followed by 35 cycles of denaturing at 94°C for 45 seconds, annealing temperature given in Table 1 (specific for each primer) for 60 seconds and extension at 72°C for 1 min, and a final extension at 72°C for 10 minutes. Then PCR results were visualized on 1.5% agarose gel.

**Table 1. SSR Markers used to assess the genetic diversity in *P. pardus*.**

| Locus | Previous study (bp) | Fragment Size (bp) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Muzaffarabad | Neelum | Hattian | Poonch | Bhimber | Kotli |
| **FCA026** | 136-154 | 138-153 | 130-149 | 137-145 | 135-143 | 140-150 | 143-152 |
| **FCA043** | 116-128 | 116-125 | 112-120 | 109-118 | 111-123 | 112-120 | 114-124 |
| **FCA075** | 103-143 | 104-141 | 108-138 | 101-137 | 109-134 | 102-132 | 103-140 |
| **FCA090** | 93-120 | 95-115 | 99-110 | 96-113 | 98-106 | 97-110 | 98-115 |

**Simple Sequence Repeat (SSR)/Microsatellite Analysis**

**Generation of binary dataand genetic diversity:** For SSR analysis we generated Bivariate 1-0 data matrix on the basis of size of bands obtained on gel and determined Allele frequencies (common alleles, rare alleles), genetic diversities (within and among populations), homogeneity of allele frequencies, total number of alleles, effective number of alleles and Shannon diversity index.We used POPGENE 32 program for calculating the total genetic diversity (Ht), genetic variation within and among populations and total population differentiation (Gst),Gene diversity index (He), the proportion of polymorphic loci (P), and the mean number of alleles per locus (A) calculated for each locus and population according to Nei (1973): $He = 1 - \sum Pi^2$ were calculated where Pi is the $i^{th}$ allele frequency.

**Genetic distance:** We performed Cluster analysis for construction of dendrogram using unweighed pair group mathematical averages (UPGMA) based on the genetic distances and genetic identity for population relationship. Then we inserted matrix of genetic distances and genetic similarities into MEGA5 program for the construction of

dendrogram and determined Polymorphic information content (PIC) based on following formula:

$PIC = 1- (\sum pi^2)$

Where Pi is the frequency of the $i^{th}$ alleles and n is the number of alleles and this value referred as heterozygosity and gene diversity (Weir,1990; Anderson *et al*., 1993).

## RESULT

**Allele frequency:** Simple Sequence repeat (SSR) analysis was carried out for which total 4 SSRs markers (FCA090, FCA043, FCA026 and FCA073)were used. Genetic diversity was calculated by the amplification of alleles per microsatellite locus, within different individuals of different populations of common leopard (*P. pardus*).

The amplified allele size ranged from 95 to 153 bps with maximum of 2 numbers of alleles amplified for each locus. The number and size of alleles of common leopard genotypes at 4 SSR loci is given in(Table 2). The highest frequency (1.0) was detected at all locus in pop-6 (Kotli), while in pop-2 Neelum only FCA090 showed allele frequency 1 and FCA026 showed lowest range of allele frequency (0.1340-0.2929) in pop-2 (Table 3).

**Table 2. Size (bp) of SSR markers amplified in different common leopard population of AJK (Raymond *et al.*, 1999)**

| Primers | Primer Sequence(5´–3´) | Initial Annealing temperature (Ta) | Optimized Ta (°C) | Amplicon (bp) | References |
|---|---|---|---|---|---|
| FCA090 | Forward | ATCAAAAGTCTTGAAGAGC | 55 | 45 | 90-111 | Menotti-Raymond *et al*., 1999. Uphyrkina*et al.*, 2001 |
| | Reverse | TGTTAGCTCATGTTCATGTGTCC | | | |
| FCA043 | Forward | GAGCCACCCTAGCACATATACC | 58 | 52 | 111-115 | Menotti-Raymond *et al*., 1999. Uphyrkina*et al.*, 2001 |
| | Reverse | AGACGGGATTGCATGAAAA | | | |
| FCA026 | Forward | GGAGCCCTTAGAGTCATGCA | 56 | 50 | 150 | Menotti-Raymond *et al*., 1999. Uphyrkina*et al.*, 2001 |
| | Reverse | TGTACACGCACCAAAAACAA | | | |
| FCA075 | Forward | ATGCTAATCAGTGGCATTTGG | 54 | 49 | 112-146 | Menotti-Raymond *et al*., 1999. Uphyrkina*et al.*, 2001 |
| | Reverse | GAACAAAAATTCCAGACGTGC | | | |

**Genetic Diversity between Loci and Population:** Genetic diversity is a commonly used indicator to assess the genetic differentiation among populations. The highest mean values of effective number of alleles (ne=1.53), Nei gene diversity index (h=0.31) and Shannon information index (I=0.45) were found in population-4 (Poonch) while population-6 (Kotli) showed the lowest values, i.e., 1.0 and 0 respectively (Table 3).

Table 4 summarizes the result of heterozygosity, genetic diversity and genetic variation between different populations of *P.pardus* species. Among four loci, FCA075 locus showed highest genetic variation that is 0.6811 while, FCA026 expressed lowest value of genetic variation (Gst) i.e. 0.1629. Mean gene flow (Nm) between six populations was found to be very low (0.8030). The value of genetic distance acquired from POPGENE-32 ranged from 0.0393 to 0.4275 (Table 5).

Highest genetic distance (0.4275) was established between the Kotli (Pop-6) and Hattian (Pop-3) populations, while the lowest between Hattian (Pop-3) and Neelum (Pop-2) populations (0.0393) of common leopard. Nei's genetic similarities between different populations were highest (0.9614) between of Hattian (Pop-3) and Neelum (Pop-2) populations while the lowest (0.6521) between Kotli (Pop-6) and Hattian (Pop-3) populations (Table 5).

Dendrogram constructed using UPGMA model divided different populations into three main clusters. Cluster I was divided into two sub groups, Pop-6 (Kotli), and Pop-5 (Bhimber). Cluster II was divided into two, Pop-2 (Neelum) and Pop-3 (Hattian) while Cluster III also contained two populations, Pop-1 (Muzaffarabad), and Pop-4 (Poonch) (Figure 1). Pop-5 (Bhimber) and Pop-6 (Kotli), as in first cluster, showed higher genetic

differentiation and separated as an isolated group from other populations and is an outlier of other main clusters. This clustering pattern showed the genetic variation between population and groups may have resulted in isolation of leopard populations due to habitat fragmentation.

**Table 3. Summary of different study constant at different SSR loci in common leopard population in AJK**

| Population | Constants | Locus | | | | Mean St. Dev |
|---|---|---|---|---|---|---|
| | | FCA026 | FCA043 | FCA075 | FCA090 | |
| Pop-1 (Muzaffarabad) (n=6) | na | 2 | 2 | 1 | 1 | 1.5±0.40 |
| | Frequency | 0.81-0.91 | 0.57-0.70 | 0.40-0.59 | 0.42-0.59 | |
| | ne | 1.42 | 1.93 | 1.0 | 1.0 | 1.51±0.41 |
| | H | 0.29 | 0.48 | 0 | 0 | 0.29±0.21 |
| | I | 0.47 | 0.67 | 0 | 0 | 0.42±0.29 |
| Pop-2 (Neelum) (n=4) | na | 2 | 2 | 2 | 1 | 1.62±0.51 |
| | Frequency | 0.13- 0.29 | 0.50-0.86 | 0.50-0.70 | 1.0-1.0 | |
| | ne | 1.30 | 2.0 | 1.70 | 1.0 | 1.41±0.43 |
| | H | 0.23 | 0.50 | 0.4 | 0 | 0.23±0.219 |
| | I | 0.39 | 0.69 | 0.60 | 0 | 0.34±0.30 |
| Pop-3 (Hattian) (n=3) | na | 2 | 2 | 1 | 2 | 1.50±0.50 |
| | Frequency | 0.18-0.42 | 0.57-0.81 | 0.18-1.0 | 0.42-0.57 | |
| | ne | 1.42 | 1.95 | 1.0 | 1.95 | 1.4±0.47 |
| | H | 0.29 | 0.48 | 0 | 0.48 | 0.22±0.24 |
| | I | 0.47 | 0.68 | 0 | 0.68 | 0.31±0.34 |
| Pop-4 (Poonch) (n=2) | na | 2 | 2 | 1 | 2 | 1.75±0.46 |
| | Frequency | 0.29-0.70 | 0.29-1.0 | 0.70- 1.0 | 0.29-0.7 | |
| | ne | 1.70 | 1.70 | 0 | 1.70 | 1.53±0.32 |
| | H | 0.41 | 0.41 | 0 | 0.41 | 0.31±0.19 |
| | I | 0.60 | 0.60 | 0 | 0.60 | 0.45±0.279 |
| Pop -5 (Bhimber) (n=2) | na | 2 | 1 | 1 | 2 | 1.37±0.51 |
| | Frequency | 0.29-0.70 | 0.70-1.00 | 0.70-1.00 | 0.29-0.70 | |
| | ne | 1.7 | 1.0 | 1.0 | 1.70 | 1.26±0.36 |
| | H | 0.41 | 0 | 0 | 0.41 | 0.15±0.21 |
| | I | 0.60 | 0 | 0 | 0.60 | 0.22±0.31 |
| Pop-6 (Kotli) (n=2) | na | 1 | 1 | 1 | 1 | 1.0±0.0 |
| | Frequency | 1.0 | 1.0 | 1.0 | 1.0 | 1.00±0.00 |
| | ne | 1.0 | 1.0 | 1.0 | 1.0 | 1.0±0.0 |
| | H | 0 | 0 | 0 | 0 | 0.00±0 |
| | I | 0 | 0 | 0 | 0 | 0.00±0.00 |

Where na= Mean number of alleles; ne= effective number of alleles; ne = Nei gene diversity index; I= Shannon information index

**Table 4: Total heterozygosity and genetic differentiation within and among different population of common leopard by SSR markers**

| Loci | Ht | Hs | Gst | Nm |
|---|---|---|---|---|
| FCA043 | 0.4638 | 0.3142 | 0.3225 | 1.0505 |
| FCA026 | 0.2111 | 0.1767 | 0.1629 | 2.5695 |
| 1FCA075 | 0.4716 | 0.1504 | 0.6811 | 0.2341 |
| FCA090 | 0.4999 | 0.2380 | 0.5240 | 0.4543 |
| Mean | 0.0314 | 0.2020 | 0.3837 | 0.8030 |

Where Ht= Total genetic diversity, Hs = G. Diversity within Populations, Gst= Diversity among population, Nm = Gene flow

**Table 5:Nei's genetic identity (above diagonal) and genetic distance (below diagonal) by microsatellites of common leopard populations**

| Populations | Muzaffarabad | Neelum | Hattian | Poonch | Bhimber | Kotli |
|---|---|---|---|---|---|---|
| Muzaffarabad | **** | 0.9213 | 0.9314 | 0.9248 | 0.76778 | 0.82 60 |
| Neelum | 0.0820 | **** | 0.9614 | 0.8919 | 0.8245 | 0.7180 |
| Hattian | 0.0711 | 0.0393 | **** | 0.8491 | 0.7664 | 0.6521 |
| Poonch | 0.0782 | 0.1145 | 0.1635 | **** | 0.6635 | 0.7528 |
| Bhimber | 0.2643 | 0.1929 | 0.2661 | 0.4102 | **** | 0.8326 |
| Kotli | 0.1912 | 0.3312 | 0.4275 | 0.2840 | 0.1833 | **** |

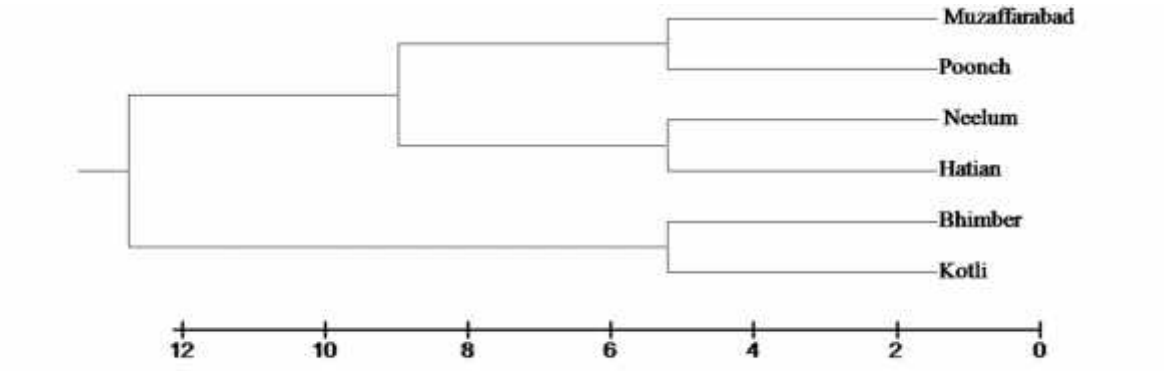


Figure: 1 Cluster analysis by 4 SSR markers in different populations of common leopard found in AJK

## DISCUSSION

Our study provides status of genetic diversity of common leopard in AJK, Pakistan. Milligan *et al*. (1994) suggest that for conservation of endangered wild species the maintenance of genetic diversity is important.It was found that all parameters of genetic diversity were consistent with each other. Shannon diversity index for band frequency and Nei's diversity index collectively explain level of genetic diversity between populations. The results of present study showed that there was allow level of polymorphism, heterozygosity and genetic diversity among six populations of *Pantherapardus* across all loci. Low level of genetic diversity shows the isolation of populations. According to Frankham (1997) isolated populations have shown low level of heterozygosity and genetic diversity.

This lower level of heterozygosity indicates a stronger inbreeding effect within populations of leopard. Inbreeding causing population fragmentation and isolations, expected to face bottleneck effect of smaller population.According toDriscoll *et al.* (2002) inbreeding play an important role on population viability causing bottleneck effect due to decreased genetic diversity and low mutation rate that persist at most genetic locus. Frankhamand Ralls (1998) suggest that Inbreeding coefficient (Fst) is more dominant factor for causing vulnerable status in natural population.

Present result showed that genetic differentiation and isolation between population causing the low level of gene flow between population. According to Petren*et al.* (2005) genetic differentiation between populations is due to genetic drift in absence of genetic flow. According toHutchison andTempleton (1999) in the absence of genetic flow, genetic drift plays important role in causing the genetic differentiation. The present results suggested no correlation between geographical distance and genetic differentiation (Fst). According to ChiapperoandGardenal (2003) restricted gene flow is due to loss of correlation between geographical distance and genetic variation due to which genetic drift increase.

Higher level genetic differentiation between populations supports our hypothesis of habitat fragmentation causing loss of genetic diversity in leopard populations. This study result indicated that genetic drift by isolating factors like barrier and fragmentations tend to decrease of genetic diversity in a species.Therefore, study of the genetic effects of population fragmentation is of central importance for conservation biology. Habitat fragmentation is possibly a great threat to the survival of populations of many mammal species (Bright,1993).

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