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Molecular Characterization and Phylogeny of *Panthera pardus* (Common Leopard) in Pakistan

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ABSTRACT

The present study was designed to investigate the evolutionary relationship of the *Panthera pardus* subspecies found in Pakistan by exploring the partial DNA sequences of mitochondrial Cytochrome c Oxidase subunit I (COI) gene. Scat samples of 15 different known leopards were collected from Nathiagali, Khyber Pakhtunkhwa, Pakistan and Azad Jammu and Kashmir, Pakistan. After amplification with specific oligos, the amplicons of partial region of COI were subjected to sequencing, and then observed for single nucleotide polymorphisms (SNPs). SNPs were observed on three loci of the COI gene as compared to reference sequence of *Panthera pardus pardus*. The specimens found in Pakistan were found to be closely related to *Panthera pardus orientalis* (Amur Leopard in South eastern Russia) and *Panthera pardus japonensis* (North Chinese Leopard), with all of these three speculated to be evolved from *Panthera pardus pardus* in not-so-far past. Our findings are helpful in ascertaining the origin and closeness of leopard subspecies in Pakistan.

INTRODUCTION

The leopard (*Panthera pardus*) is one of the most agile, adaptable and widely distributed felid (Nowell and Jackson, 1996). These wild cats play a vital ecological role as the apex predators and also have an effect on smooth functioning of the ecosystem (Pitman, 2012). Leopards can be found inhabiting rain forests, deserts, tropical and temperate regions of the world (Seidensticker and Lumpkin, 1991). This geographically widespread species is further divided in 27 currently recognized trinomial designations (Uphyrkina *et al*., 2001) though the subspecies taxonomic classification is controversial (Miththapala *et al*., 1996). Their population status varies and is not uniform across Asia and Africa (Nowell and Jackson, 1996). It is also proposed by some studies that the subspecific taxonomy of the species should be revised (Miththapala *et al*., 1996) as classical phenotypic approaches and craniometric analyses are not sufficient to explain the genetic diversity of such a widely distributed species. The IUCN Red List of Threatened Species illustrates the same fact in their taxonomic notes (Miththapala *et al*., 1996; Uphyrkina *et al*., 2001).

In Pakistan, common leopard (*Panthera pardus*) serves as a flagship species, found in the northern region of Pakistan from Margalla Hills National Park, Murree Hills Forest, Ayubia National Park (KP) and adjoining forest of Khyber-Paktunkhwa and Azad Jammu and Kashmir. According to World Wildlife Fund (WWF), Pakistan, four subspecies have been reported from Pakistan. Though no documented or genetic evidences sum up the types of subspecies found in Pakistan, historic taxonomic data reveal following subspecies to be reportedly

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seen in different regions of Pakistan: Indian leopard (*P.p. fusca*), Persian leopard (*P.p saxicolor*), Balochistan leopard (*P. p. sindica*) and Kashmir leopard (*P.p. millardi*).

The wildlife researchers in the country have characterized the common leopard only on the basis of anatomical and some phenotypic characteristics which include the variations in coat color pattern, forehead spot pattern variation, number and spacing of rosettes found on the muzzle (Mithathapala *et al*., 1996). There is no baseline information about the molecular characterization and evolutionary status of common leopard in Pakistan. The taxonomic issues related to common leopard can be addressed by employing DNA barcoding which has emerged as a powerful tool for species identification. In this regard, mitochondrial DNA harbors few peculiar biological properties which render it more reliable and suitable genetic marker for biodiversity and evolutionary analysis (Brown *et al*., 1979). The most commonly and dependably used DNA barcode site for animal taxa comprises of about 650 bps of COI gene which covers almost 40% of total genes (Yu *et al*., 2011). It is also known as high level diversity marker and has been employed in molecular systematic (Hebert *et al*., 2003).

Keeping in view the need to address the pertaining issues related to this near threatened subspecies, the present study was designed to genetically characterize Common Leopard in Pakistan and to determine its evolutionary relationship with similar species and genera. The study was aimed to explore the genetic consistency of Common Leopard which can help in laying basis to conserve this near-threatened species.

MATERIALS AND METHODS

Scat samples from common leopards (n=15) were obtained from Nathiagali, Khyber Pakhtunkhwa, Pakistan and Azad Jammu and Kashmir, Pakistan with the help of WWF, Pakistan. The surface of the scat samples was scraped to obtain sloughed epithelial cells found along the length of the intestine. DNA extraction was carried out using Exgene™ Stool DNA mini following the manufacturer's protocols with a few modifications. DNA quantification was done with the help of Nanodrop (Thermoscientific, Wilmington, USA) as already employed (Goldshtein *et al*., 2009). All DNA samples were adjusted at the same concentration (50 ng/µL). Conserved regions of mitochondrial genome including Cytochrome c Oxidase subunit I (COI) gene was selected to design primers from conserved flanking regions using mitochondrial DNA reference sequences (Accession No. NC_010641.1) (Table I). The designed primers were synthesized from Gene Link, USA.

PCR for DNA of samples was performed using respective primers for amplification of COI genes for all samples processed. Concentration and quantity of negative and positive sense primers was 1 uL (10 pM each). Other ingredients of recipe included 1 uL of cDNA, 3 uL of 1X reaction buffer, 3 uL of 5mMdNTPs, 3U of Taq DNA Polymerase and PCR grade water. To avoid non-specific binding across the templates, stringent thermal cycling conditions were programmed with 30 cycles of denaturation at 95°C for 15 seconds, annealing at 54°C for 20 seconds and extension at 72°C for 45 seconds. Amplicons were visualized on UV light illuminator to see specific sized product. The precipitated PCR products were sequenced in both directions using di-deoxy chain termination method (Sanger *et al*., 1977). Sequencing was done with the help of automated sequence analyzer from 1st Base, Malaysia. Data analysis was performed using software BioEdit, Chromas and MEGA.

Phylogenetic tree was inferred using COI gene sequences of the *Panthera pardus* subspecies samples collected and tested from Pakistan compared to COI genomic sequences of other *Panthera pardus* subspecies and *Panthera* spp. and related feline genera/species.

RESULTS AND DISCUSSION

Estimates of evolutionary divergence among COI genome sequences based upon number of base substitutions per site was conducted using MEGA6 (Tamura *et al*., 2013). Analyses were conducted using Minimum Evolution Method (Tamura *et al*., 2004). The analyses involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 544 positions in the final dataset. PCR of the COI gene resulted in amplification of 543bp fragment (Fig. 1). Homology analysis of COI gene revealed that three unique positions (639 G>A; 888 G>A, and 894 C>T) can be used to identify the *Panthera pardus* subspecies present in Pakistan from rest of the sub-species.

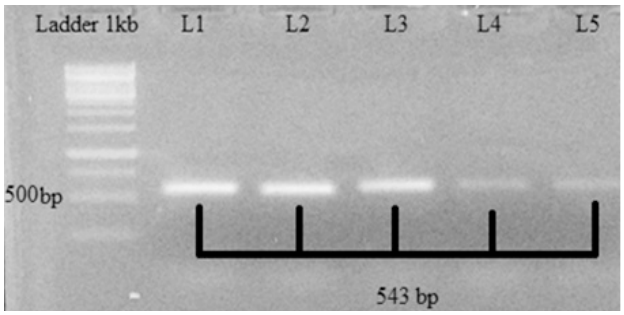

Fig. 1. PCR amplification of COI gene (543bp size).

Table I.- Primers for COI gene.

Primer Name	Primer Length (bp)	Product size (bp)	Primer sequence (5'-3')
COI F	20	543	CTGCTGTATTGCTACTCCTA
COI R	20	543	GCTCCTATTGACAGTACGTA

Table II.- Estimates of Evolutionary Divergence between Sequences with respect to COI genome sequence.

Panthera pardus **(Pakistan)**												
Panthera pardus japonensis (KJ866876.1)	0.0024											
Panthera pardus pardus (EF551002.1)	0.0049	0.0024										
Panthera pardus orientalis (KP202265.1)	0.0024	0.0000	0.0024									
Panthera_uncia (KP202269.1)	0.0582	0.0554	0.0554	0.0554								
Panthera_tigris (EF551003.1)	0.0915	0.0883	0.0883	0.0883	0.0671							
Panthera_leo leo (KF776494.1)	0.0625	0.0596	0.0596	0.0596	0.0372	0.0597						
Puma_concolor (NC_016470)	0.1101	0.1068	0.1034	0.1068	0.0939	0.0875	0.0858					
Paradoxurus hermaphroditus (KC894743.1)	0.1317	0.1280	0.1298	0.1280	0.1302	0.1375	0.1280	0.1215				
Felis_catus (NC_001700.1)	0.1067	0.1034	0.1001	0.1034	0.0860	0.1032	0.0858	0.0838	0.1268			
Neofelis nebulosa (NC_008450.1)	0.1067	0.1034	0.1034	0.1034	0.0939	0.0967	0.0921	0.1243	0.1307	0.1236		
Panthera_onca (NC_022842.1)	0.0611	0.0582	0.0582	0.0582	0.0360	0.0552	0.0086	0.0844	0.1302	0.0840	0.0871	
Prionailurus bengalensis (KP246843.1)	0.0969	0.0937	0.0936	0.0937	0.0907	0.0748	0.0826	0.0701	0.1196	0.0654	0.0969	0.0778

A phylogenetic tree (Fig. 2) constructed on the basis of amplified COI gene sequences, other species of Genus *Panthera* and some other related feline species revealed the evolutionary status of the big cats under study.

The estimates of evolutionary divergence indicate that the *Panthera pardus* subspecies found in Pakistan are not too far evolutionarily divergent from other subspecies of *Panthera pardus* at least on the basis of COI genome sequence. Common leopard found in Pakistan shares its clad with *Panthera pardus japonensis* which is indigenous to Northern China and *Panthera pardus orientalis* (Amur Leopard), native to the Primorye region of southeastern Russia and the Jilin Province of northeast China. *Panthera pardus orientalis* and *Panthera pardus japonensis* exibhit almost complete homology with respect to COI genome sequence and share a common clad with *Panthera pardus* subspecies found in Pakistan, with an evolutionary divergence value of 0.0024. The observations of relative

times show that these three subspecies of *Panthera pardus* evolved at the same time from *Panthera pardus pardus*, which is the basic subspecies of common leopard.

Panthera pardus pardus is the subspecies found in Africa and evolution of classical trinomially designated subspecies of *Panthera pardus* is speculated to be from main stock of *Panthera pardus* (subspecies pardus) through a land bridge before continental drift. As the continents drew apart, the evolving climatic conditions faced by geographically separated populations forced them to evolve according to their respective habitats (Janczewski *et al*., 1995). Or, in other words, the fittest, according to Darwin, survived resulting in genetic divergence from the main stock. The same fact is evident in comparative studies of genetic diversity between *Panthera pardus pardus* and geographically isolated island subspecies of *Panthera pardus* (Meijaard, 2004; Miththapala *et al*., 1996). The emigration of specimens of common leopard from Africa might also be due to movement of hordes of mercenaries accompanied with warlords in prehistoric times, when keeping feral/wild animals was a common practice for high ranked armed personnel.

The closest related specieS in Genus *Panthera* was found to be the snow leopard (*Panthera uncia*), big cat specie indigenous to high altitude northern areas of Pakistan, the modern taxonomic classification of whom is at a similar status as that of *Panthera pardus* subspecies in Pakistan. The sequencing results of COI gene plotted on molecular diversity clock had relative time spacing value of 0.01 for each event of genetic diversity. *Panthera pardus* (Pakistan), *Panthera pardus orientalis, Panthera pardus japonesis* and *Panthera pardus pardus* were closest recent forms of genetic diversity (Relative Time 0.0). Snow leopard shares a large proportion of common genome with *Panthera pardus* and speciation of the species might occur due to habitat differentiation and resultant morphological changes in not-so-far past. Similarly, *Panthera leo* (Lion) and *Panthera onca* (Jaguar) were established as the closest cousins of *Panthera pardus* (Common Leopard) diverged from a common feline ancestor at a value 0.03 of relative time from current status of genetic diversity (0.00), while oldest common ancestor of all morphologically related organisms, compared and analyzed in this study, resides at relative time value between 0.06 to 0.07 (Fig. 2).

The hypotheses of evolutionary divergence of *Panthera pardus* subspecies is also supported by convergent evolution exhibited by *Panthera onca* (Jaguar). The body coat of both (*Panthera pardus* and *Panthera onca*) allows these species to function well in their respective habitats by providing a perfect camouflage.

It is proposed on the basis of this study that an extensive study may be initiated for recognition of types of *Panthera pardus* subspecies found in Pakistan and the establishment of their distinct taxonomic positions based on advanced molecular markers. The same may be conducted for the genetic relationship of *Panthera pardus* with nearby species like *Panthera uncia* (Snow Leopard) as respective data are currently unavailable. Modern taxonomic tools may help in the efforts to recognize and conserve precious wildlife species to ensure the continuity of their beautiful existence on this planet.

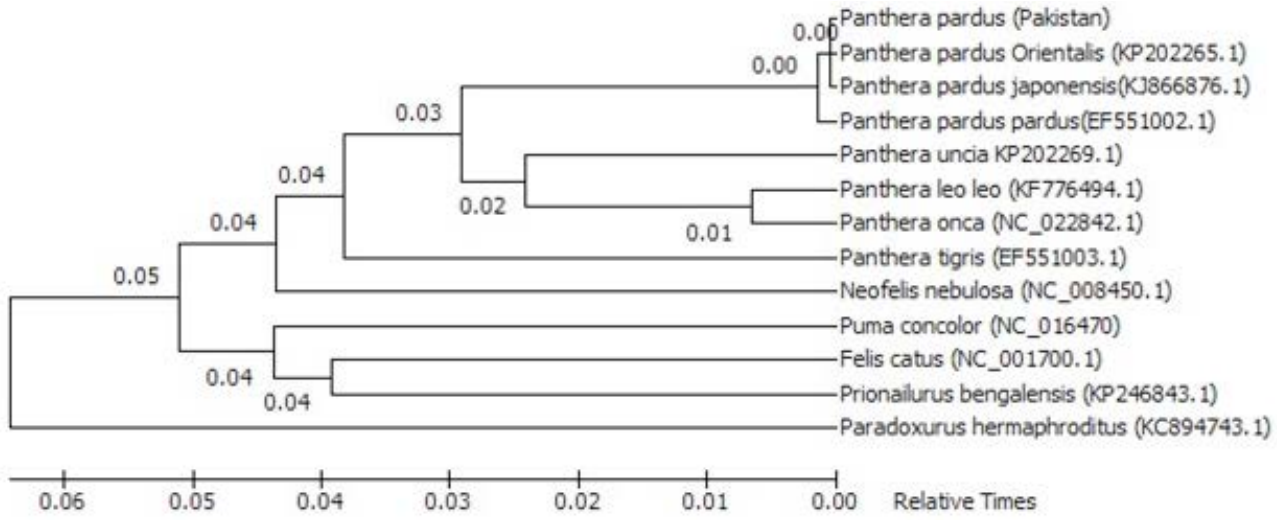

Fig. 2. Phylogenetic tree of *Panthera pardus* spp. with respect to COI gene sequence.

CONCLUSION

Current study provides an insight on the characterization of Common Leopard in Pakistan, which is ranked as 'Critically Endangered' (WWF-Pakistan) and 'Vulnerable' (IUCN Red List) specie. This study may prove helpful for devising conservation strategies for preservation of this flagship specie, along with laying a basis for further research on wildlife phylogenetics and associated conservation efforts.

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Conflict of interest statement

We declare that we have no conflict of interest.

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