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SEQUENCE DIVERSITY AND PHYLOGENETIC ANALYSIS IN PAKISTANI SPOTTED DEER *(Axis axis)*

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Spotted deer or "Chital" are one of the most common wild herbivore found in forests. The main objective of the study was to assess the genetic variations in mitochondrial *cytochrome-b* gene, mitochondrial *cytochrome-c* gene and mitochondrial *d-loop* region in Pakistani spotted deer. DNA was extracted from fecal samples of unrelated animals of spotted deer from different localities. PCR primers were designed by Primer software and amplification of gene was done by Polymerase Chain Reaction. The PCR products were sequenced. Variations in nucleotide were identified by alignment. Blast 2 sequences, Clustal W, MEGA 3, Bioconductor in "R" were used for analysis. Multidimensional scaling plot of mitochondrial *cytochrome-b, c* and *d-loop* region, Pair Wise Evolutionary Distance and Phylogenetic Tree was constructed. Variable sites in *Cytochrome-b*, Cytochrome-c and d-loop region were identified in spotted deer. Allele frequency of all variations was calculated and low mutant allele frequency was observed. High homozygosity was observed in all spotted deer and differences were very low. This is the first report on molecular genetics of spotted deer from Pakistan. The finding of this research is prerequisite for future research.

Keywords: Sequence, diversity, phylogenetic, spotted deer

INTRODUCTION

The Spotted Deer or Chital (*Axis axis*) is only Cervid within genus Axis. Three other species that were part of this genus are now grouped in *Hyelaphus* on the basis of their genetic evidence. *Axis axis* is also known as chital deer, spotted deer or axis deer. *Axis axis* is very common in Indian subcontinent. They live in herds. Antlers of spotted deer are lyre-shaped and way of howling is primitive as compared to other species of deer. Spotted deer is very common in forests of India, Sri Lanka, Nepal, Bangladesh, Bhutan, but it has very small population in Pakistan (Duckworth *et al*., 2008).

Population of spotted deer has decreased during last two centuries because of human activities and the uncontrolled use of natural resources. Under the influence of human activities in present times, a lot of animal species have become extinct or they are in the danger of extinction. This extinction of animal species not only has ecological but also economic and social loss for human being. Small population size due to excessive hunting results in loss of genetic diversity that is required for acclimatization of a population in changing environmental conditions (Thevenon *et al.*, 2004).

Different plannings and actions are oriented towards restoration of such species that had plentiful populations in the past. One of the most common actions in this regard is translocation of individuals from viable populations. This causes loss of genetic diversity. So, present day attention is focused to avoid or reverse the loss of genetic diversity in such populations or to establish populations with high genetically diversity in former areas.

In present era of molecular genetics, Cytochrome- *b, c* and *d-loop* region of mitochondria are potent markers that can be used for characterization of different genetic resources (Goldstein and Pollock, 1997). Molecular data on Genetic diversity and phylogenetic analysis studies in Pakistani spotted deer is rare. In this context there was need to explore the genetic architecture variation and phylogenetic relations within Pakistani populations of spotted deer.

MATERIALS AND METHODS

DNA was extracted from fecal samples of unrelated animals of spotted deer. DNA extraction method was followed as described by Zhang *et al.* (2006). Quantification of extracted DNA samples were done with the help of Nanodrop (Thermoscientific, Wilmington USA). Reference sequences of complete mitochondrial genome including *d loop*, *cytochrome b* and *Cytochrome c* regions for *Axis axis* (Accession No NC_020680) were retrieved from NCBI (www.ncbi.nlm.nih.gov). Sequences were aligned by the Molecular Evolutionary Genetics Analysis (MEGA)

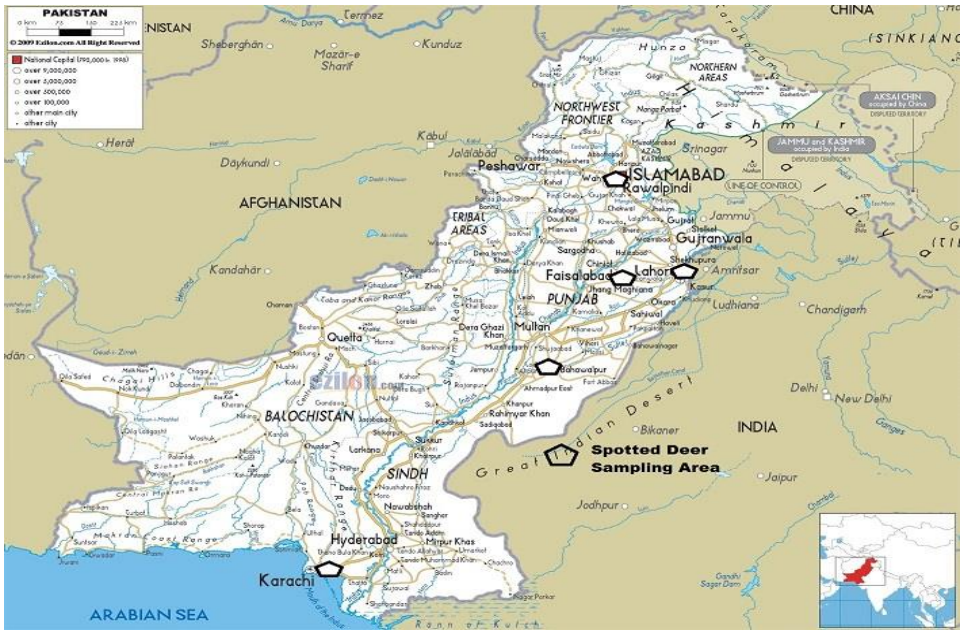

Figure 1. Map of Pakistan showing selected areas for sampling of Spotted deer.
The figure was modified from http://www.ezilon.com

software. Close homology among the sequences of each loci allowed designing of a common pair of suitable primers for each locus, hence three pairs of primers were designed for complete amplification of three loci. Moreover, two additional forward primers were also designed for the purpose of sequencing of cytochrome c. All primers were designed using the primer blast of NCBI (www.ncbi.nlm.nih.gov) and synthesized from Genelink, USA.

Each pair of primer was used in amplification of each DNA sample of spotted deer. Precipitated PCR products were sequenced using dye labeled dideoxy chain termination sequencing using ABI Genetic Analyzer 3130 XL (Applied Biosystem Inc., Foster city, CA, USA).

Bioinformatics tools, Blast 2 sequences, Clustal W (Thompson *et al*., 1994), MEGA 3, Bioconductor in R (Gentleman *et al*., 1999), were used for sequence analysis.

RESULTS

***Cytochrome-b gene based phylogenetic analysis*:** Variations were identified in *Cytochrome-b* gene of spotted deer (Table 3). Regarding allele frequency, no heterozygosity was observed. Multidimensional scaling (MDS) plot was created by 'R' and figured out by using 1st and 2nd dimensional transformations. Evolutionary distance matrix was done by sequences analyses and the matrix was then utilized to plot

Table 1. Sampling details of all Animals samples used in this study.

Sr. No.	Samples	Source	Google coordinates	No. of samples
1	AA1, AA10	Bahawalpur Zoo, Bahawalpur	29°24'8.7"N 71°40'54.5"E	2
2	AA6, AA7	Bahria Town Lahore	31°18'51.5"N 74°12'11.7"E	2
3	AA4, AA5	Bahria Town Rawalpindi	33°29'45.2"N 73°6'20.3"E	2
4	AA11, AA12	Basti Bahadurpur Multan	30°15'27.9"N 71°29'48.2"E	2
5	AA22	Shabbir Abad, Jhang	31°23'29.9"N 72°24'33.1"E	1
6	AA14, AA15	Karachi zoo, Karachi	24°52'32.9"N 67°1'22.7"E	2
7	AA16, AA17	Lahore safari park, Lahore	31°22'53.9"N 74°12'41.6"E	2
8	AA19, AA18	Lahore Zoo, Lahore	31°33'22.7"N 74°19'34.0"E	2
9	AA20, AA2	Lohi Bher Wildlife Park Rawalpindi	33°57'49.5"N 73°11'93.1"E	2
10	AA13	Mukhiana, Jhnag	31°23'14.9"N 72°27'50.7"E	1
11	AA3	Peerowal Khanewal	30°20'22.7"N 72°2'2.4"E	1
12	AA23, AA24, AA25	Rana Resort Head Balloke	31°11'25.9"N 73°52'32.6"E	3
13	AA8, AA21	Wildlife farms Lahore	31°23'5.5"N 74°14'8.5"E	2
14	AA9	Wildlife Park Vehari	30°2'14.7"N 72°21'2.6"E	1
Total				25

Table 2. List of primer sequences.

Primer Name	Locus	PRIMER SEQUENCE (5' TO 3')	Product size (bp)	*TA* (°C)
CytoB-F	*Cytochrome-b*	GTCATTCAACTACAAGAACACTA	1289	51°C
CytoB-R	*Cytochrome-b*	TAAATAGAACTTCAGCTTTGGG		51°C
CytoC-F1	*Cytochrome-c*	GCTTCAATCTACTTCTCCCG	1761	53°C
CytoC-R	*Cytochrome-c*	GTGGTTATGATGTTGGCTTG		53°C
CytoC-F2	*Cytochrome-c*	CACCTRGCAGGYGTCTC	1242	53°C
CytoC-F3	*Cytochrome-c*	GATCACCTGCYATAATATGAGC	650	53°C
DLF	*d- Loop*	AGCCTCACTATCAACACCCA	1020	54°C
DLR	*d- Loop*	CACATAGGTTTGGTCCCAGC		54°C

MDS (Fig. 2). Computational BH87 model was used to generate distance profile for *Cytochrome-b* sequence of spotted dear. Figure 3 was generated by using the genetic distance dataset of mitochondrial genomic region of *Cytochrome-b*. The Neighbor joining phylogenetic tree was constructed by using maximum likelihood method implemented in a desktop application named as MEGA-6 (Fig. 4).

Table 3. Polymorphisms identified in *Cytochrome-b* gene of *Axis axis.*

No.	Base Position (with reference to NC_020680)	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	14221	A→G	0	1
2	14598	C→T	0	1
3	15099	A→G	0	1
4	15199	A→G	0	1
5	15219	T→C	0	1
6	15249	T→C	0	1
7	14221	A→G	0.80	0.20
8	15299	A→G	0.56	0.44
9	15372	G→A	0.92	0.08
10	15439	T→C	0.92	0.08
11	15449	T→C	0.56	0.44

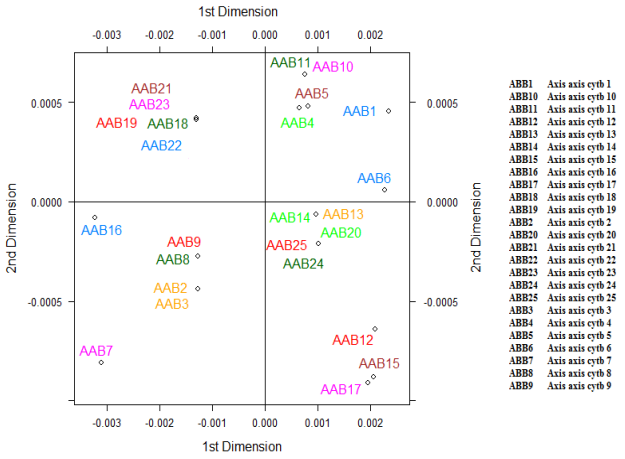

Figure 2. Multidimensional scaling plot of mitochondrial *cytochrome-b* gene for *Axis axis.*

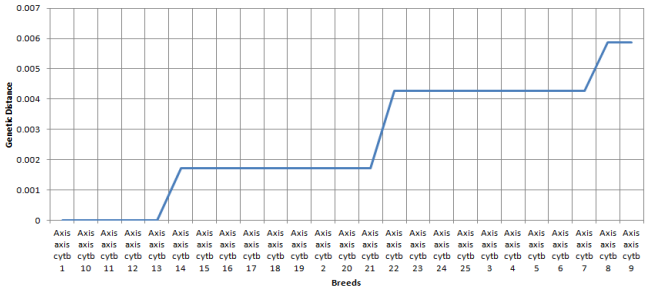

Figure 3. *Cytochrome-b* based genetic variation plot of *Axis axis.*

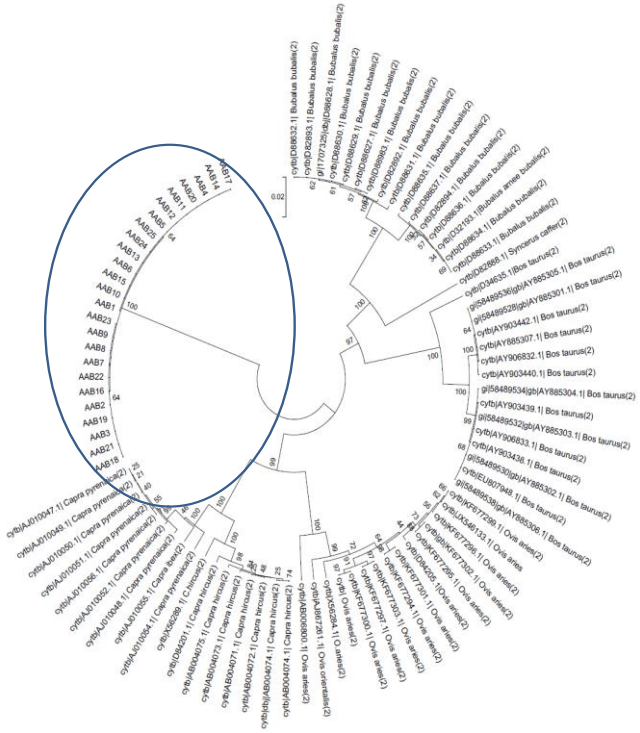

Figure 4. Phylogenetic tree (Circular) of *Cytochrome-b* region of *Axis axis.*

***Cytochrome-c gene based analysis*:** Variations in *Cytochrome-c* gene of spotted deer were identified (Table 4). No heterozygosity was observed. Allele frequency "1"

represents that all samples were monomorphic. MDS plot (Fig. 5), pair wise evolutionary distance (Fig. 6) and phylogenetic tree (Fig. 7) was calculated as described earlier.

Table 4. Identified Polymorphisms in *Cytochrome-c* gene of *Axis axis.*

No.	Base Position (with reference to NC_020680)	Change in Nucleotide (Wild to Mutant)	Allele Frequencies A	B
1	5414	T→A	0	1
2	5441	G→A	0	1
3	6684	T→C	0	1
4	5414	T→A	0.84	0.16
5	5441	G→A	0.84	0.16

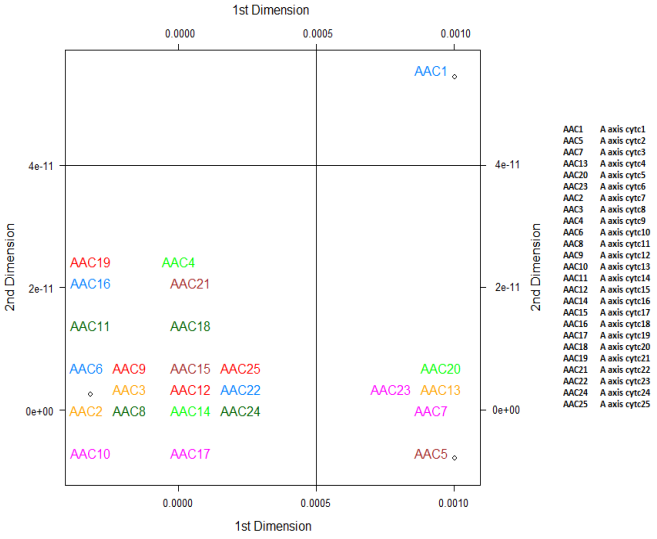

Figure 5. Multidimensional scaling plot of *Cytochrome-c* gene for *Axis axis.*

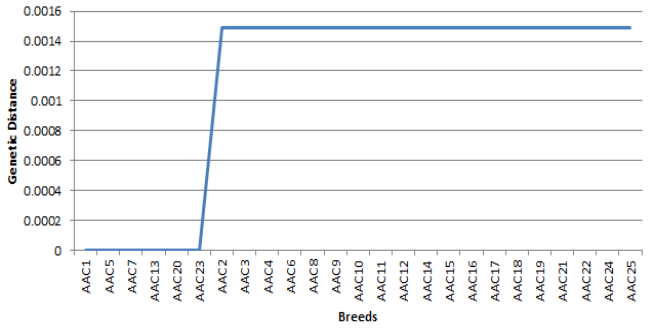

Figure 6. *Cytochrome-c* based genetic variation plot of *Axis axis.*

***Mitochondrial d-loop region based analysis*:** Sequence variations were identified in mitochondrial *d-loop* region (Table 5). No heterozygosity was observed. MDS plot (Fig. 9), pair wise evolutionary Distance (Fig. 8) and phylogenetic tree (Fig. 10) was constructed.

Table 5. Polymorphisms in mitochondrial *d-loop* of *Axis axis.*

No.	Base Position (with reference to NC_020680)	Change in Nucleotide (Wild to Mutant)	Allele Frequencies A	B
1	15647	C→T	0	1
2	15673	C→T	0	1
3	15676	C→T	0	1
4	15679	T→C	0	1
5	15723	G→A	0	1
6	15763	T→C	0	1
7	15472	T→A	0.92	0.08
8	15610	A→G	0.92	0.08
9	15701	A→G	0.84	0.16
10	15783	C→T	0.92	0.08
11	15843	C→T	0.84	0.16
12	15934	C→T	0.84	0.16
13	15997	T→C	0.92	0.08
14	15997	C→T	0.92	0.08
15	16141	G→A	0.92	0.08
16	16264	T→C	0.80	0.20

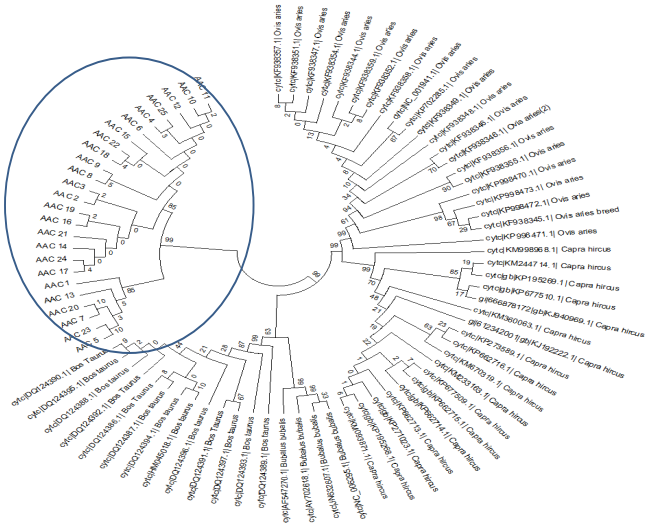

Figure 7. Phylogenetic tree (Circular) of *Cytochrome-c* region of *Axis axis.*

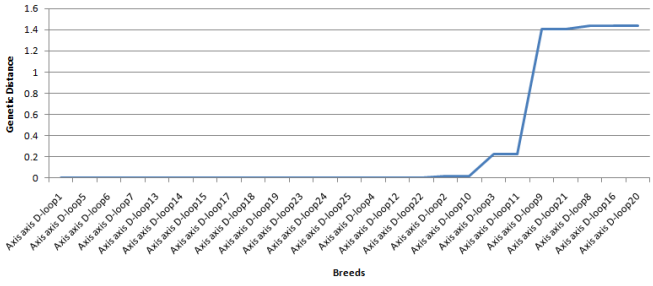

Figure 8. *d-loop* region based genetic variation plot of *Axis axis.*

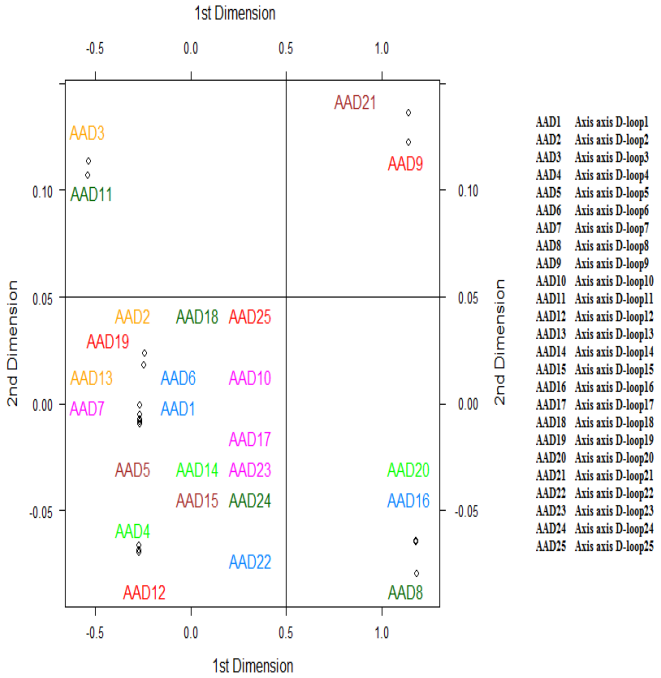

Figure 9. Multidimensional scaling plot of mitochondrial *d-loop region* for *Axis axis.*

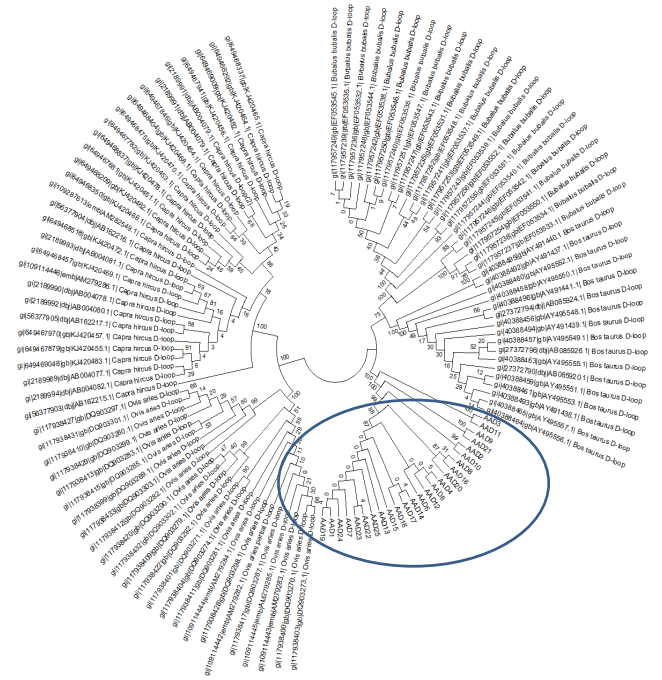

Figure 10. Phylogenetic tree (Circular) of *d-loop* region of *Axis axis.*

Table 6. *Cytochrome-b, cytochrome-c and d-loop* region based evolutionary analyses.

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
[1]																				
[2]	0.00368																			
[3]	0.02546	0.02348																		
[4]	0.00141	0.00339	0.02518																	
[5]	0.00085	0.00283	0.02461	0.00057																
[6]	0.00057	0.00311	0.02489	0.00085	0.00028															
[7]	0.00141	0.00283	0.02461	0.00113	0.00057	0.00085														
[8]	0.00481	0.00566	0.02687	0.00396	0.00396	0.00424	0.00396													
[9]	0.01160	0.01414	0.02178	0.01245	0.01188	0.01216	0.01188	0.01103												
[10]	0.00339	0.00028	0.02376	0.00311	0.00255	0.00283	0.00311	0.00594	0.01443											
[11]	0.02518	0.02376	0.00028	0.02489	0.02433	0.02461	0.02489	0.02716	0.02207	0.02348										
[12]	0.00113	0.00368	0.02546	0.00028	0.00085	0.00057	0.00141	0.00424	0.01273	0.00339	0.02518									
[13]	0.00509	0.00594	0.02716	0.00424	0.00424	0.00453	0.00368	0.00028	0.01132	0.00622	0.02744	0.00453								
[14]	0.00113	0.00255	0.02433	0.00085	0.00028	0.00057	0.00028	0.00368	0.0116	0.00283	0.02461	0.00113	0.00396							
[15]	0.00057	0.00311	0.02489	0.00141	0.00085	0.00113	0.00085	0.00424	0.01103	0.00339	0.02518	0.0017	0.00453	0.00057						
[16]	0.00396	0.00651	0.02772	0.00424	0.00424	0.00453	0.00481	0.00085	0.01075	0.00622	0.02744	0.00453	0.00113	0.00453	0.00396					
[17]	0.01216	0.01358	0.02122	0.01188	0.01132	0.0116	0.01132	0.01047	0.00057	0.01386	0.0215	0.01216	0.01075	0.01103	0.0116	0.01132				
[18]	0.0017	0.00311	0.02489	0.00028	0.00085	0.00113	0.00085	0.00368	0.01216	0.00339	0.02518	0.00057	0.00396	0.00057	0.00113	0.00453	0.0116			
[19]	0.00113	0.00255	0.02433	0.00085	0.00028	0.00057	0.00028	0.00368	0.0116	0.00283	0.02461	0.00113	0.00396	0.00000	0.00057	0.00453	0.01103	0.00057		
[20]	0.00339	0.00368	0.02546	0.00368	0.00311	0.00283	0.00368	0.00707	0.01499	0.00339	0.02518	0.00339	0.00736	0.00339	0.00396	0.00736	0.01443	0.00396	0.00339	
[21]	0.0611	0.06167	0.0826	0.06082	0.06025	0.06054	0.06082	0.06365	0.07072	0.06139	0.08232	0.0611	0.06393	0.06054	0.0611	0.06393	0.07016	0.0611	0.06054	0.06025

***Three genes combined sequence analysis*:** Multidimensional scaling plot of combined sequences of cytochrome-b, cytochrome-c and d-loop was generated and figured out by 1st and 2nd dimensional transformations which showed symmetrical variation of genetic distance values (Fig. 11). Pair wise evolutionary distance (Table 6) and estimates of evolutionary divergence between sequences is shown in Fig. 12. Phylogenetic tree by maximum likelihood method was constructed as shown in Figure 13.

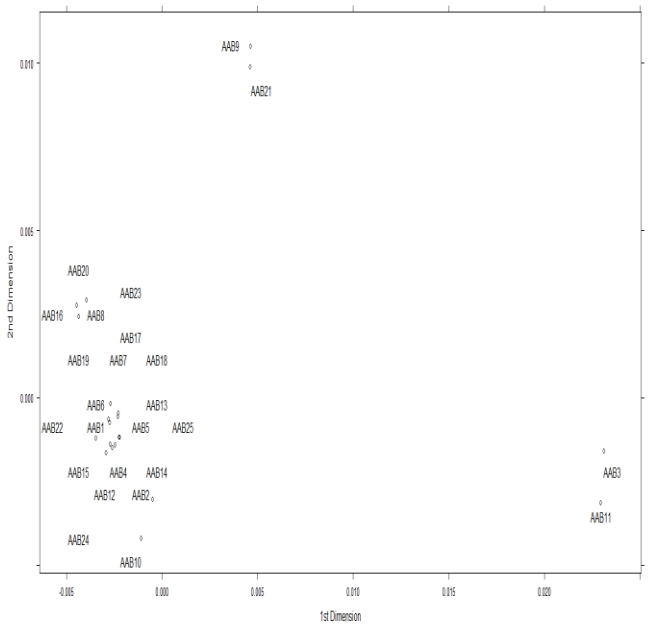

Figure 11. Multidimensional scaling plot of mitochondrial genomic *cytochrome-b, cytochrome-c and d-loop region (collectively)* for *Axis axis.*

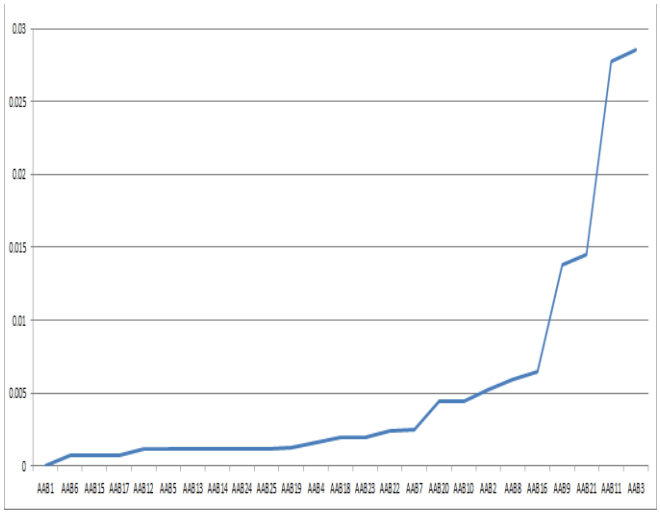

Figure 12. *Cytochrome-b, cytochrome-c and d-loop* region (collectively) based genetic variation plot of *Axis axis.*

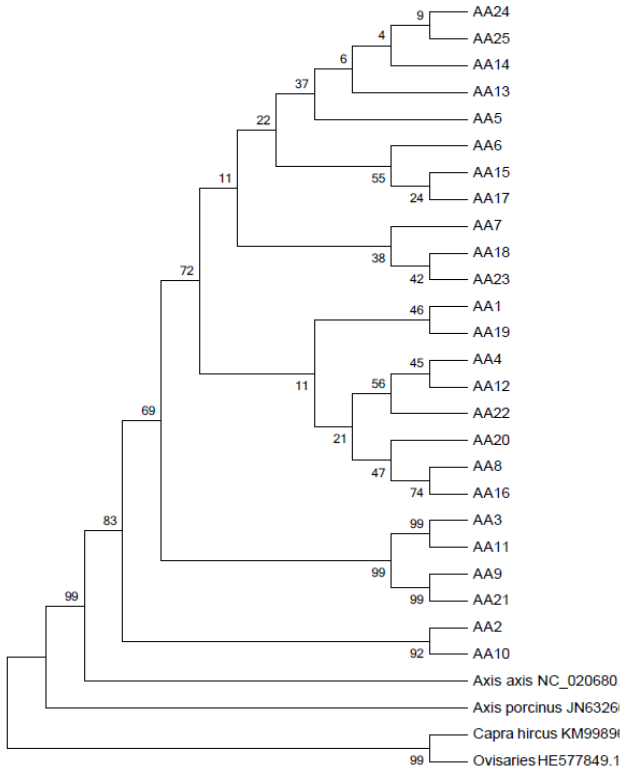

Figure 13. Phylogenetic tree (Rectangular) of *Cytochrome-b, cytochrome-c and d-loop* region (collectively) of *Axis axis.*

DISCUSSION

Pakistan is faced with many threats of environment degradation for species ecosystem. Diverse climatic and ecological conditions, wide latitudinal and altitudinal geographical location of Pakistan makes it favorite territory for mammals. Invention of modern tools of land cultivation, illegal hunting and rapid urbanization led to devastating decrease in wild animals. Currently, only limited patches at different locations of Pakistan have small populations of Cervidae especially *Axis axis.* So this specie is on their way towards possible scantiness especially from Indo-Pak region. Molecular markers provide the initial picture for evaluation of genetic variation (Allendorf *et al*., 2010). Molecular marker based phylogenetic analysis of Pakistani buffalo, goat, sheep, camel (Babar *et al*., 2014; Hussain *et al*., 2009, 2013, 2015 have previously been reported but wildlife species especially deer species data is scarce.

Genome conservation plays important role in survival of the species and the ecosystem. For the purpose of genetic information for spotted deer in Pakistan, mitochondrial *cytochrome-b*, *cytochrome-c* and *d-loop* region have been used to determine genetic diversity and genetic structure which can be exploited in deriving information on phylogenetic relationships (Feral, 2006; Mwacharo *et al*.,

2006; Giovambattista *et a1*., 2001). DNA sequence based techniques are useful for assessing genetic variability within and among populations (Haig, 1998). The conservation of wild endangered species is dependent on maintenance of genetic variations (Crozier, 1992; Lynch and Milligan, 1994) which is an indication of variation in genomes.

Variation pattern statement represent that the samples were extracted from individuals of the same species, because of either species misidentification (human error) or imperfect taxonomy (Hassanin *et al*., 2012). Results illustrate normal distribution patterns of all four species but overall tendency was observed towards homozygosity.

A total of eleven variable sites were observed in *Cytochrome-b* gene of *Axis axis*. Six variations were monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. Transition substitution was found on all sites. All individuals were homozygous. Allele frequency of mutant allele was found low. Cytochrome-c gene (1544 bp fragment) of *Axis axis* was analysed and five variable sites were observed. Out of these, three variations were monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of three transitions and two transversions. Allele frequency of all variations was calculated and low mutant allele frequency was observed. No heterozygous individuals were found.

Polymorphisms in *d-loop* region of spotted were identified. Out of sixteen variable sites, six variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 15 transitions and one transversion. Allele frequency of all variations was calculated and very low frequency of mutant allele was observed. As no heterozygous individuals were found so allelic frequency and genotypic frequency was same. Multidimensional scaling (MDS) plot of mitochondrial *cytochrome-b, cytochrome-c* and *d-loop region* for *Axis axis* was generated. Single gene and three genes combined sequences based MDS plot was generated. Overlap was found in the overall genetic profile of the *Axis axis*.

Genetic variation is a base material for animal survival, which acts as the genetic source for prediction of future in conservation that leads to the conservation of animals, to analyze the status of conservation. Molecular markers plays initial guide for evaluation of the genetic variation (Allendorf *et al*., 2010). Therefore, the information on this level of genetic variation is prerequisite for designing effective strategy in future effective practices for conservation (Crandall *et al*., 2000). However, further genomic investigations should be carried out at larger scale.

***Conclusion*:** These results indicate that populations were significantly different from each other. Genetic variability and phylogenetic relationship within and between groups was evaluated based on polymorphic loci from *cervidae*. The level of heterozygosity was relatively comparable in all animals. The highest homozygosity was observed in all spotted deer and differences were very low.

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