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Molecular Phylogeny and Diversity Analysis of Hog Deer (*Axis porcinus*) in Pakistan

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Authors' Contribution
AN and MEB conceived and designed the study. GA, MST and RZI did sampling and genome extraction. GA and MJ amplified the markers. AN, MEB, MJ, TH and WS analyzed the data. AN and GA wrote the article. GA and AN contributed equally in this article.

Key words
Mitochondrial, Phylogenetic, Pakistani hog deer.

ABSTRACT

Many species of mammals have declined within the past two centuries due to human caused disturbances and the unsustainable use of natural resources. Molecular methods have an important role in phylogeny and diversity analysis. In Pakistan hog deer (*Axis porcinus*) is an important wild species belongs to the family Cervidae. The hog deer is one of the least studied species at molecular level in Pakistan. The present study was planned to investigate its molecular phylogeny and diversity analysisusing mitochondrial *cytochrome b*, *cytochrome c* and *d-loop region*. Samples were collected from different localities. After DNA extraction and quantification, amplification of gene was done by polymerase chain reaction. PCR products were sequenced bi-directionally, aligned and single nucleotide polymorphisms were identified. Bioinformatics tools were applied for construction of phylogenetic tree and genetic diversity analysis. Lower genetic diversity was observed. The finding of this research is prerequisite for future research.

INTRODUCTION

Hog deer (*Axis porcinus*) is member of the family Cervidae. It is found in grasslands along the rivers. With slight variation in preference regarding habitat, hog deer prefer to live in flood plain grass lands, coastal grasslands, grasslands dominated by blady grass (Imperata cylindrica) and alluvial floodplain, lightly wooded and also mountain areas in India, Nepal, Thailand, China, Vietnam and Bangladesh (www.wildlifeofpakistan.com).

In Pakistan, hog deer are limited to forests along the rivers, grasslands and especially in those areas having dense grass with scattered plants of *Saccharum spontaneum, Saccharum munja* and *Tamarix dioica*. Hog deer are native to the Indus eco-region and its habitat exists up to Attock. Indus river forests reserves in Sind have good number of its population. The animal is breeding in captivity at the Khirthar National Park. Chashma Barrage Wildlife Sanctuary, Head Islam/Chak Kotora Game Reserve, Lal Suhanra National Park Bahawalpur, Wildlife Sanctuary of Taunsa Barrage and Rasool Wildlife Sanctuary are important habitats of Hog deer (Arshad *et al*., 2012).

Most imperious molecular technique used for the characterizations that detect the genetic variation at DNA level opened the unique dimensions. DNA polymerization is more reliable and ethic approach to define the breed and species as they are not affected by environmental fluctuations (Adebambo *et al*., 2003). It was investigated about twenty years ago that mitochondrial DNA is a helpful marker for phylogeoraphy research and surprisingly molecular biologist are continuously isolating different species on the basis of unique mitochondrial loci analysis. In comparison to molecular data the mitochondrical loci proved to be much sensitive indicators for mammalians species identifications. Molecular genome has captured by molecular biologists for characterization and extensively used as molecular clocks (Bromham, 2003), even can be used where no fossils records are available. As a molecular clock it has a potential to define the time of divergence between closely related species and finally depict the molecular distance among the species.

For phylogenetic studies and species identification of different animals, *cytochrome b* along with other mitochondrial DNA markers has been used frequently in recent times (Hsieh *et al*., 2001). In this study different mitochondrial DNA regions were sequenced to identify the polymorphisms in *Axis porcinus* families and their phylogenetic relationships with other animals

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was constructed.

Table I.- Sampling details.

Sample ID	Source	Google coordinates
AP24, AP4	Bahawalpur Zoo, Bahawalpur	29°24'8.7"N 71°40'54.5"E
AP22, AP5	Bahria Town Lahore	31°18'51.5"N 74°12'11.7"E
AP10, AP12	Bahria Town Rawalpindi	33°29'45.2"N 73°6'20.3"E
AP16	Basti Bahadurpur Multan	30°15'27.9"N 71°29'48.2"E
AP21	Shabbir Abad, Jhang	31°23'29.9"N 72°24'33.1"E
AP19	Changa Manga, Kasur	31°5'19.3"N 73°57'44.7"E
AP3, AP8	Gatwala Wildlife Breeding Centre, Faisalabad	31°28'42.7"N 73°12'36.7"E
AP9, AP11	Gulshan-e-Iqbal Park, Lahore	31°30'46.2"N 74°17'31.2"E
AP17, AP20	Lahore safari park, Lahore	31°22'53.9"N 74°12'41.6"E
AP23, AP2	Lahore Zoo, Lahore	31°33'22.7"N 74°19'34.0"E
AP6, AP7	Lohi Bher Wildlife Park Rawalpindi	33°57'49.5"N 73°11'93.1"E
AP13, AP15	Peerowal Khanewal	30°20'22.7"N 72°2'2.4"E
AP18, AP25	Head Balloke Raavi River	31°11'25.9"N 73°52'32.6"E
AP14	Wildlife Park Vehari	30°2'14.7"N 72°21'2.6"E
AP1	Taunsa Barrage Wildlife Sanctuary	30°30'46.4"N 70°50'58.9"E

MATERIALS AND METHODS

Sampling was done from zoos, wildlife forms and captive breeding centers at different places of Pakistan. Details of sampling area with Google coordinates are given in Table I. Fecal samples were collected from above regions. Name and code of each animal was marked on each sample. Fecal DNA was extracted (Zhang *et al*., 2006) and quantified through Nano Drop 2000/2000c (Thermo scientific USA). Reference sequences of *Axis porcinus* (Accession No. JN632600) was taken from NCBI (www.ncbi.nlm.nih.gov). Sequences were aligned by the Molecular Evolutionary Genetics Analysis (MEGA) software to design primers from loci of interest. All primers were designed using the primer blast of NCBI (www.ncbi.nlm.nih.gov). Pair of primer was used in amplification of each DNA sample of *Axis porcinus*. PCR and sequencing methods was followed as described by Nadeem *et al*. (2013). Bioinformatics tools, Blast 2 sequences, Clustal W (Thompson *et al*., 1994), MEGA 3 (Kumar *et al*., 2001), Bioconductor in R (Gentleman *et al*., 2004), were used for data analysis.

Table II.- Identified polymorphisms in *cytochrome b* gene of *Axis porcinus*.

Base position (with reference to JN632600)	Change in Nucleotide (Wild to Mutant)	Allele frequencies	
		A	B
14297	A→G	0	1
14522	G→A	0	1
14733	A→G	0	1
14871	T→C	0	1
15295	G→A	0	1
14297	G→A	0.60	0.40
14402	A→G	0.72	0.28
14532	C→T	0.84	0.16
14875	T→C	0.60	0.40
15143	A→G	0.60	0.40
15149	G→A	0.92	0.08
15207	T→C	0.92	0.08
15232	C→T	0.76	0.24

Table III.- Polymorphisms in *Cytochrome c* gene of *Axis porcinus*

Base Position (with reference to JN632600)	Change in Nucleotide (Wild to Mutant)	Allele frequencies	
		A	B
6547	C→T	0	1
6781	T→C	0	1
5475	A→G	0.80	0.20
5501	A→G	0.84	0.16
5628	G→A	0.80	0.20
5804	G→A	0.84	0.16
5808	C→A	0.72	0.28
5858	C→T	0.80	0.2
5940	G→T	0.80	0.2
5965	A→G	0.92	0.08
6426	T→C	0.72	0.28
6585	G→A	0.92	0.08

RESULTS AND DISCUSSION

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 *Axis porcinus* of Pakistan. Sequences

of mitochondrial *cytochrome b* and *cytochrome c* and mitochondrial *d-loop*, regions for *Axis porcinus* was retrieved from NCBI (www.ncbi.nlm.nih.gov) for primer designing. Mitochondrial regions of interest were amplified and sequenced bi-directionally by Big Dye™ Terminator on ABI 3130XL Genetic analyzer.

Polymorphisms were identified in *cytochrome b*, *cytochrome c* gene and *d-loop* region (Table IV). Nill heterozygosity was observed. Allele frequency "1" represent that all samples were monomorphic. MDS plot, pair wise evolutionary distance and phylogenetic tree was calculated.

After single gene based analysis, collectively sequences of three genes (c*ytochrome* b, c*ytochrome c* and *d-loop*) based multidimensional scaling plot was generated. Plot was figured out by using 1st and 2nd dimensional transformations showing symmetrical variation of genetic distance values in MDS plot. DNA sequences of *cytochrome b, cytochrome c andd-loop*region (combined sequence) of animals under study were routed for phylogenetic analyses.

In *Axis porcinus*, 1139 bp fragment sequenced of mitochondrial c*ytochrome* b gene was analysed. A total of thirteen variable sites were observed (Table II). Out of these, five variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 13 transitions. Low frequency of mutant allele and no heterozygous individuals was observed.

Table IV.- Identified polymorphisms in mitochondrial *d-loop* region of *Axis porcinus*.

Base Position(with reference to JN632600)	Change in Nucleotide (Wild to Mutant)	Allele frequencies	
		A	B
15547	T→C	0	1
15582	T→C	0	1
15639	A→G	0	1
15697	G→A	0	1
15731	A→G	0	1
15747	T→G	0	1
15852	C→T	0	1
15597	G→A	0.80	0.20
15737	A→G	0.92	0.08
15859	G→A	0.84	0.16
15891	C→T	0.96	0.04
15919	T→C	0.80	0.2
15995	C→G	0.84	0.16
16059	C→T	0.92	0.08
16106	C→T	0.92	0.08
15189	T→C	0.84	0.16
16243	C→T	0.84	0.16
16283	A→G	0.96	0.04
16314	A→G	0.84	0.16

Table V.- *Cytochrome b, cytochrome c* and *d-loop* region (collectively) based Evolutionary analyses of *Axis porcinus.*

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
[1]																							
[2]	0.0017																						
[3]	0.00254	0.00424																					
[4]	0.00367	0.00311	0.00396																				
[5]	0.00311	0.0048	0.00283	0.00452																			
[6]	0.00254	0.00198	0.00452	0.00339	0.00509																		
[7]	0.00198	0.00028	0.00396	0.00283	0.00452	0.0017																	
[8]	0.00141	0.00311	0.00113	0.00452	0.00396	0.00339	0.00283																
[9]	0.00254	0.00198	0.00452	0.00113	0.00509	0.00226	0.0017	0.00339															
[10]	0.00113	0.00283	0.00367	0.0048	0.00198	0.00367	0.00311	0.00254	0.00367														
[11]	0.00198	0.00367	0.0017	0.00226	0.00226	0.00396	0.00339	0.00283	0.00283	0.00311													
[12]	0.00311	0.00254	0.00339	0.0017	0.00283	0.00283	0.00226	0.00396	0.00283	0.00311	0.00283												
[13]	0.00113	0.00283	0.00254	0.0048	0.00424	0.00254	0.00311	0.00141	0.00367	0.00226	0.00311	0.00424											
[14]	0.00028	0.00198	0.00226	0.00339	0.00283	0.00226	0.0017	0.00113	0.00226	0.00141	0.0017	0.00283	0.00141										
[15]	0.00113	0.0017	0.00367	0.0048	0.00311	0.00367	0.00198	0.00254	0.00367	0.00113	0.00311	0.00424	0.00226	0.00141									
[16]	0.00311	0.00254	0.0017	0.00226	0.00339	0.00283	0.00226	0.00283	0.00283	0.00424	0.00226	0.0017	0.00424	0.00283	0.00424								
[17]	0.00254	0.00424	0.0017	0.00226	0.00339	0.00452	0.00396	0.00226	0.00339	0.00367	0.00113	0.00283	0.00254	0.00226	0.00367	0.00339							
[18]	0.00424	0.00367	0.00396	0.00226	0.00226	0.00396	0.00339	0.00509	0.00283	0.00424	0.00339	0.0017	0.00537	0.00396	0.00537	0.00226	0.00452						
[19]	0.00254	0.00424	0.0017	0.00339	0.00226	0.00452	0.00396	0.00226	0.00452	0.00254	0.00226	0.00283	0.00367	0.00226	0.00254	0.00226	0.00226	0.00452					
[20]	0.00226	0.0017	0.0048	0.00254	0.00537	0.00141	0.00198	0.00367	0.00141	0.00339	0.00311	0.00311	0.00339	0.00254	0.00339	0.00311	0.00367	0.00424	0.0048				
[21]	0.00057	0.00226	0.00311	0.00424	0.00254	0.00311	0.00254	0.00198	0.00311	0.00057	0.00254	0.00254	0.0017	0.00085	0.0017	0.00367	0.00311	0.00367	0.00311	0.00283			
[22]	0.00311	0.00254	0.00283	0.00113	0.00339	0.00283	0.00226	0.00396	0.0017	0.00424	0.00113	0.0017	0.00424	0.00283	0.00424	0.00113	0.00226	0.00226	0.00339	0.00198	0.00367		
[23]	0.0599	0.06103	0.06075	0.06188	0.06132	0.06132	0.06075	0.06075	0.06132	0.06103	0.06019	0.06132	0.06103	0.05962	0.06103	0.06075	0.06132	0.06188	0.06132	0.0616	0.06047	0.06075	
[24]	0.00678	0.00791	0.00763	0.00819	0.00819	0.00819	0.00763	0.00763	0.00819	0.00791	0.00706	0.00763	0.00791	0.0065	0.00791	0.00763	0.00763	0.00876	0.00763	0.00848	0.00735	0.00763	0.06019

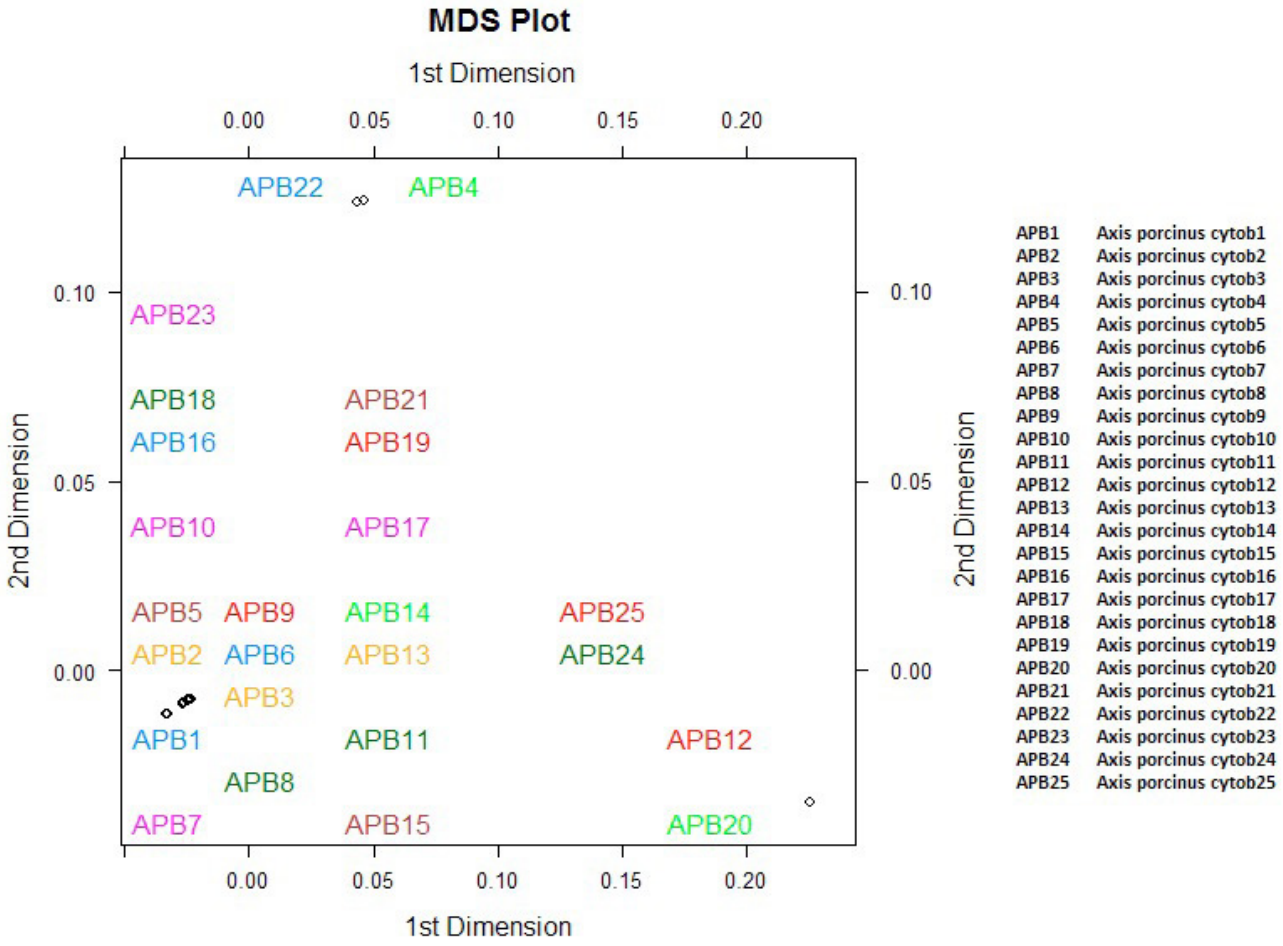

Fig. 1. Multidimensional scaling plot of mitochondrial c*ytochrome b* for *Axis porcinus*.

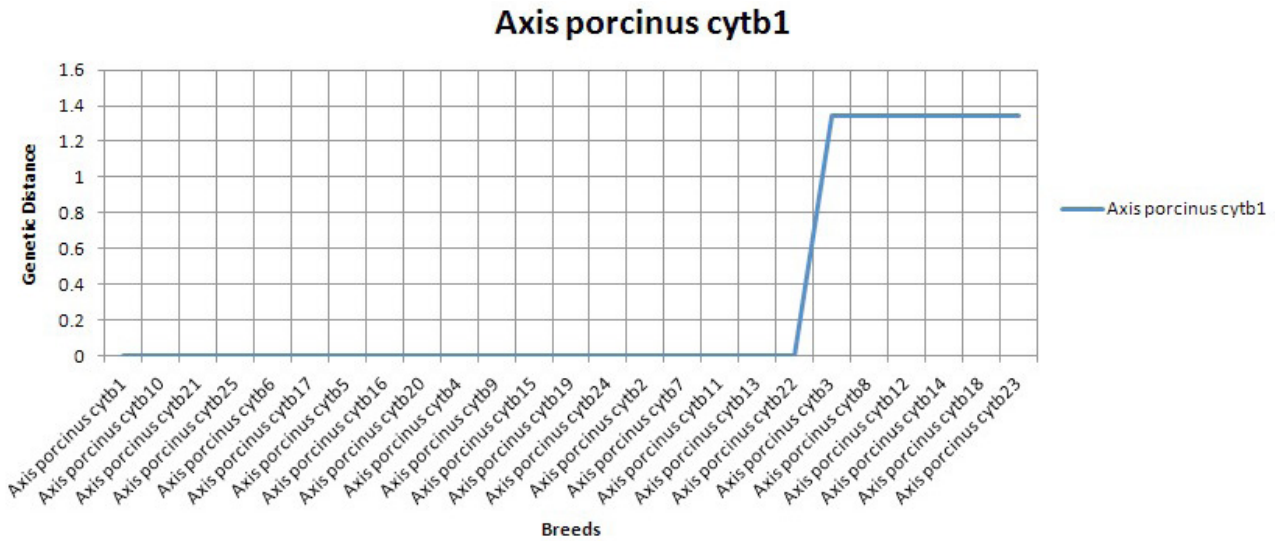

Fig. 2. *Cytochrome b* based genetic variation plot of *Axis porcinus*.

Fig. 3. Phylogenetic tree (Circular) of cytochrome *b* region of *Axis porcinus*.

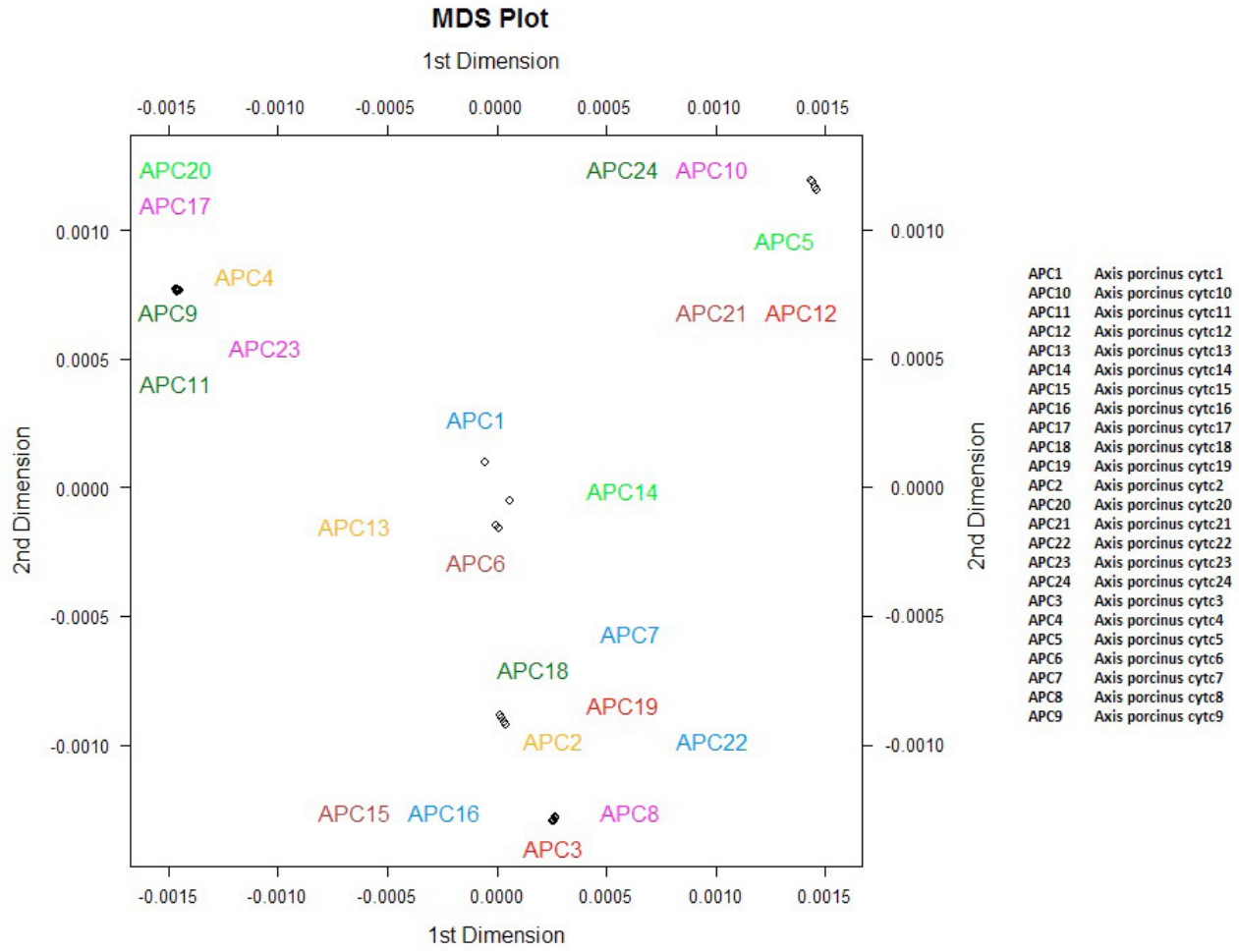

Fig. 4. Multidimensional scaling plot for *Axis porcinus* based on *cytochrome c*.

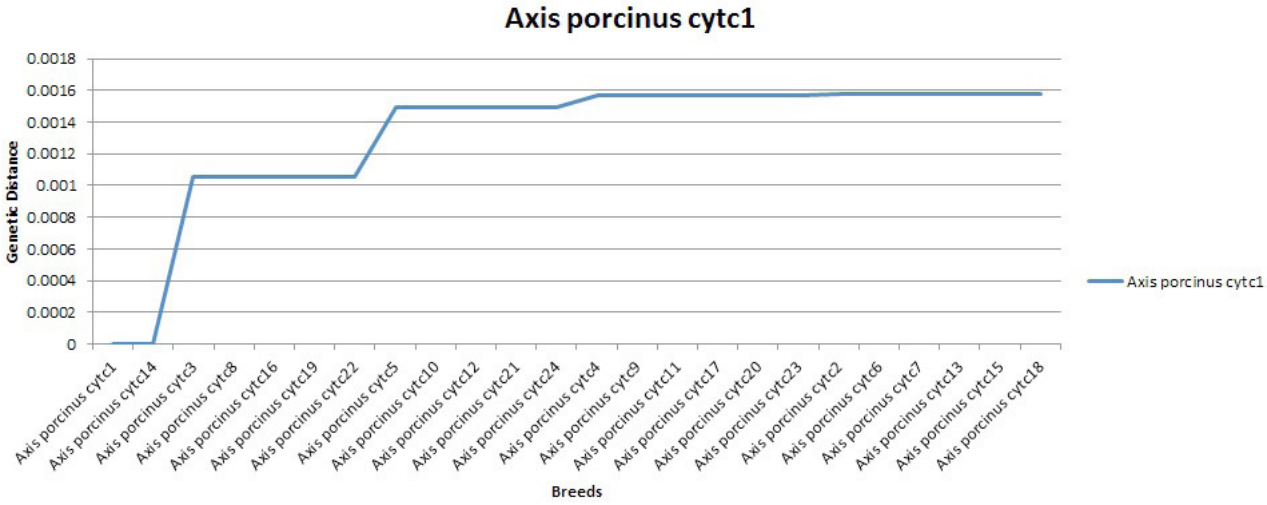

Fig. 5. *Cytochrome c* based genetic variation plot of *Axis porcinus*.

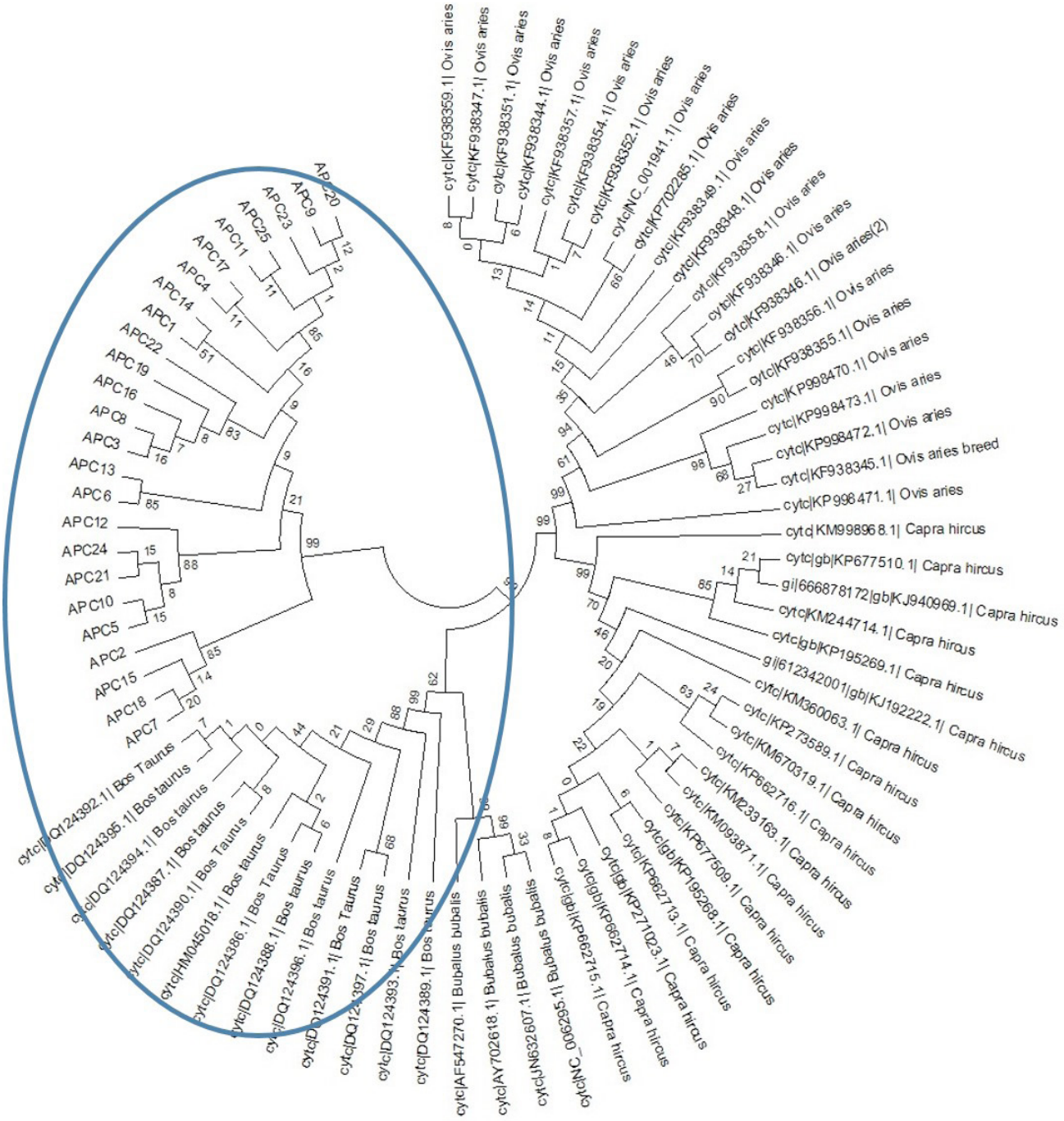

Fig. 6. Phylogenetic tree (Circular) of *cytochromec* region of *Axis porcinus.*

In the c*ytochrome* c gene of the mitochondrial DNA, 1526 bp fragment from Axis porcinusindividuals was analysed. A total of twelve variable sites were observed. Out of these, two variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. Eleven transitions and one transversion was observed. 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.

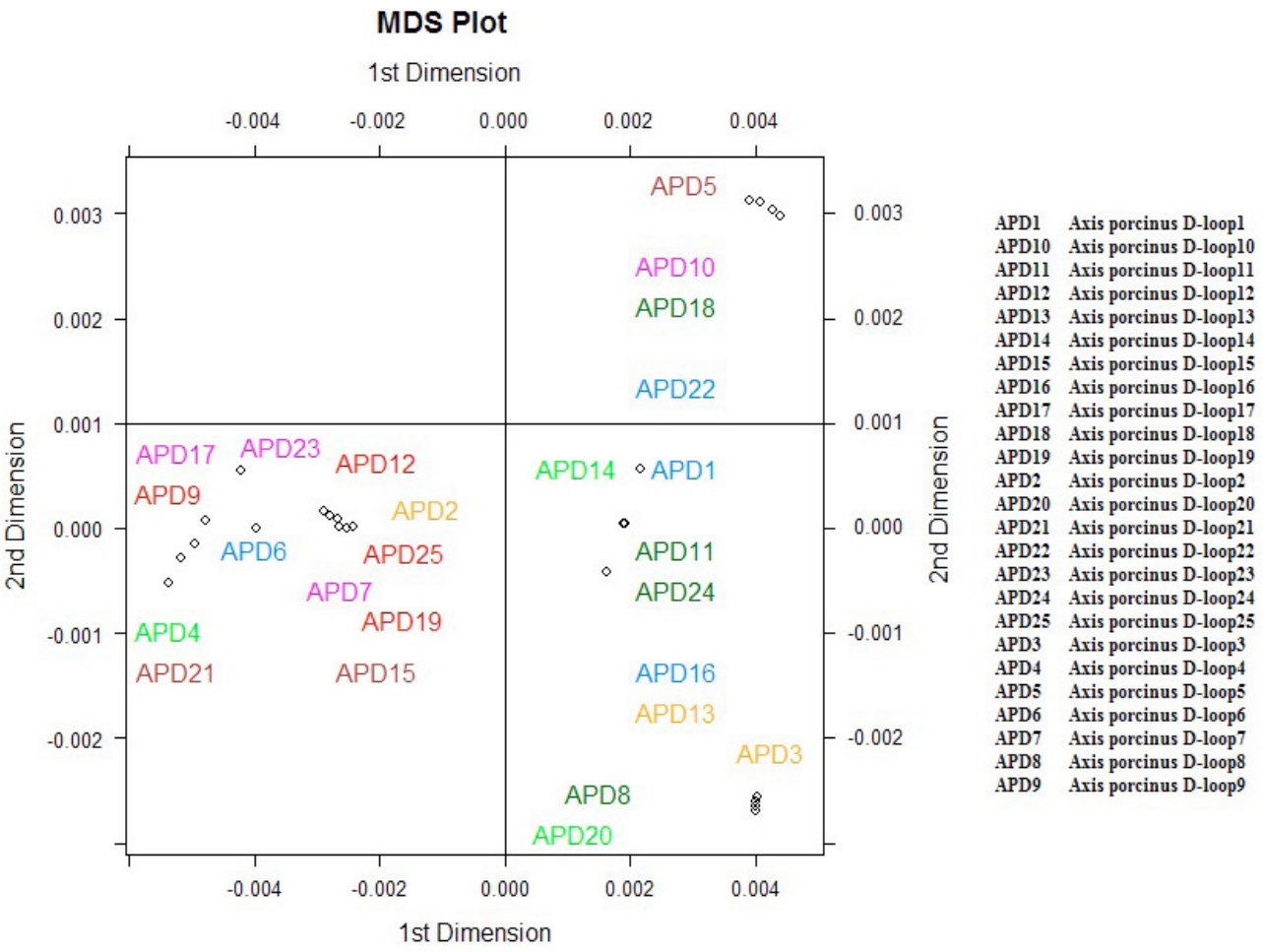

Fig. 7. Multidimensional scaling plot of d-*loop region* for *Axis porcinus.*

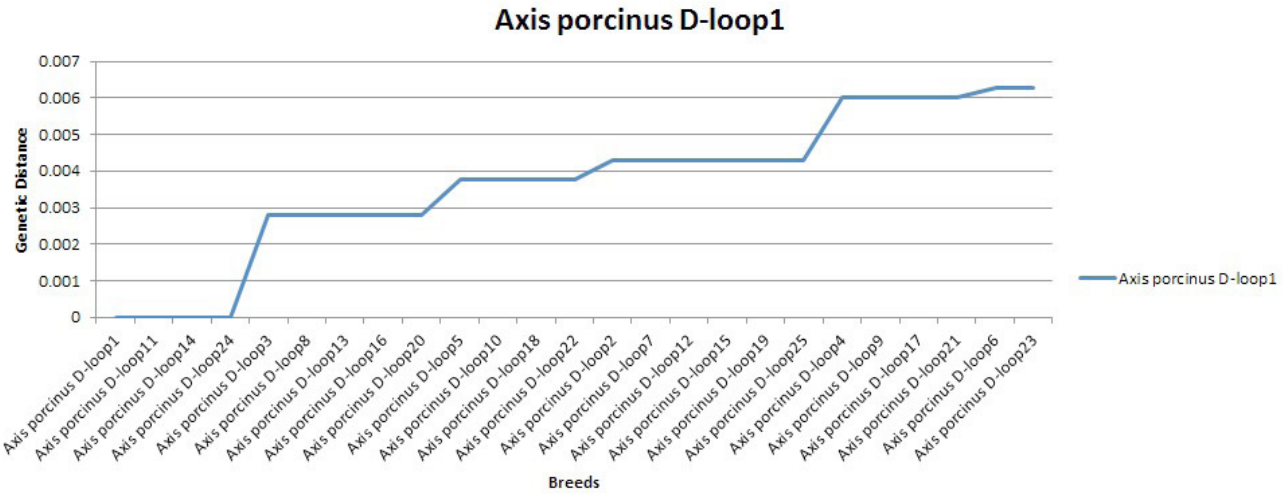

Fig. 8. *d-loop* region based genetic variation plot of *Axis porcinus.*

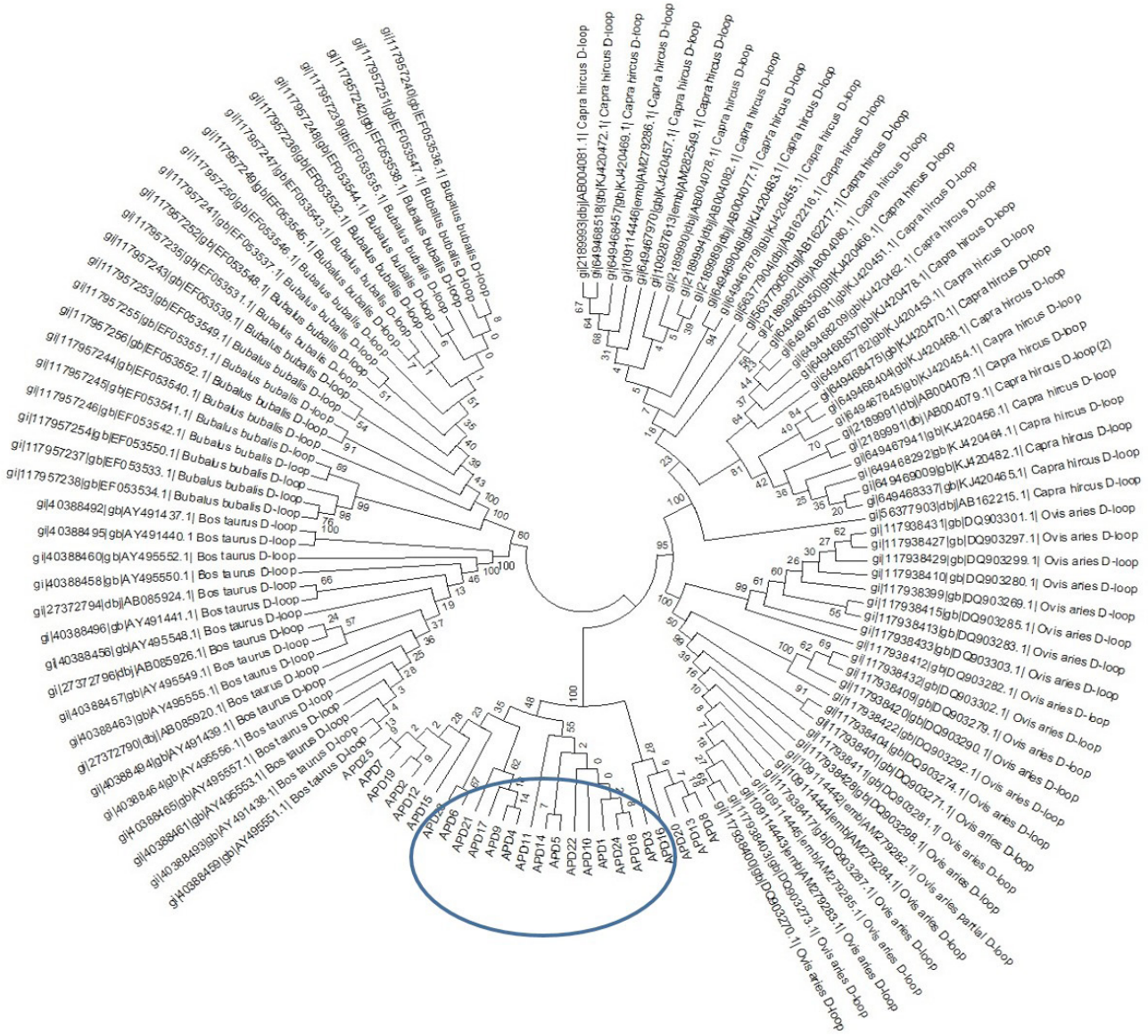

Fig. 9. Phylogenetic tree (Circular) of d-*loop* region of *Axis porcinus.*

Mitochondrial*d-loop* region of *Axis porcinus* (688 bp fragment) individuals was sequenced and analysed. A total of nineteen variable sites were observed (Table III). Out of these, seven variations were monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 18 transitions and one transversion. No heterozygous individuals were found.

Multidimensional scaling (MDS) plot of mitochondrial *cytochrome b* (Fig. 1) and genetic variation plot (Fig. 2), *cytochrome c* (Fig. 4) and *d-loop region* (Fig. 7) for *Axis porcinus* was generated. Genetic variation plot based on *cytochrome c* (Fig. 5) and *d- loop region* (Fig. 8) for *Axis porcinus* was presented. Single gene and three genes combined sequences based MDS plot was generated for each species of deer (Fig. 10). The greater clustering of the *Axis porcinus* samples also indicates lower genetic variability. *Cytochrome b*, *cytochrome c* and *d-loop region* (collectively) based Evolutionary analyses of *Axis porcinus* is shown in Table V. *Cytochrome b* (Fig. 3), *cytochrome c* (Fig. 6) and *d-loop* region (Fig. 9) based phylogenetic was constructed. Phylogenetic tree of *cytochrome b, cytochrome c andd-loop* region (collectively) of *Axis porcinus* was also constructed and presented in Figure 12.

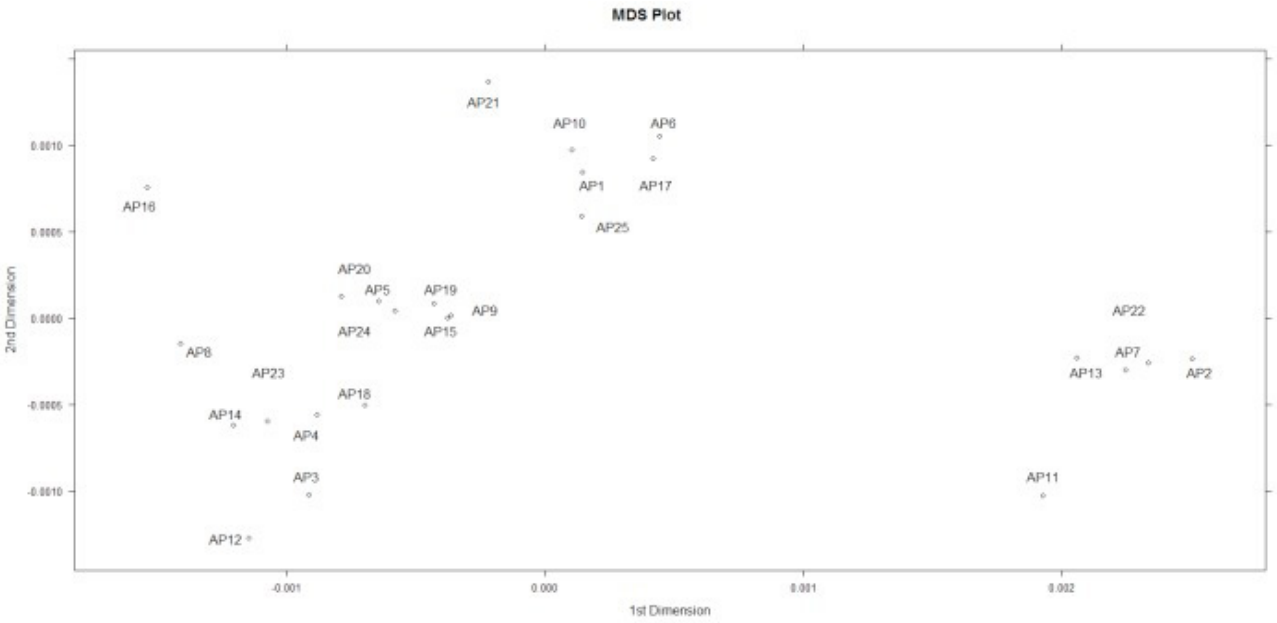

Fig. 10. Multidimensional scaling plot of mitochondrial genomic *cytochrome b, cytochrome c and d-loop region* for *Axis porcinus*.

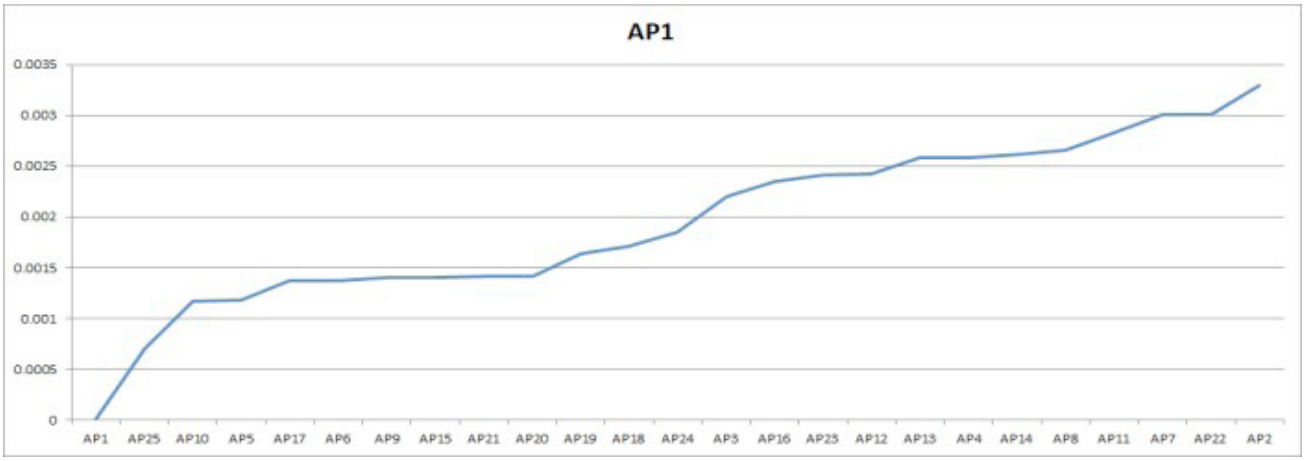

Fig. 11. *Cytochrome b, cytochrome c andd-loop* region (collectively) based Genetic variation plot of *Axis porcinus.*

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). Phylogenetic analysis of Pakistani livestock breeds such as buffalo, goat, sheep, camel (Babar *et al*., 2014; Hussian *et al*., 2009, 2013a, 2013b, 2015; Ahmed *et al*., 2014) have previously been reported but wildlife species data is scarce.

Cytochromeb and *d-loop* parts of mitochondria are potent markers that can be used for characterization of different genetic resources (Goldstein and Pollock, 1997).

According to Babar *et al*. (2015), as for as diversity between species is concerned, mitochondrial DNA show fast and distinguishing mutations but on the other hand those are very rare with in species. In addition to above mentioned utilization, *cytochromeb* and *d-loop* parts of mitochondria have their role as markers in solving forensic cases relating to conflicts of parentage. The reported mitochondrial genome sequence will facilitate conservation, population, management, and genetic epidemiology research in cervidae while also enabling conservation genomics in the endangered species of deer.

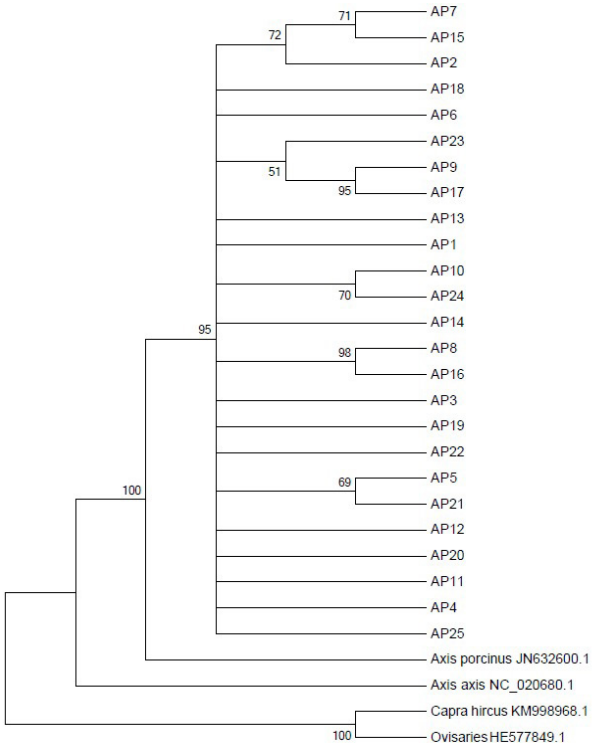

Fig. 12. Phylogenetic tree (Rectangular) of *cytochrome b, cytochrome c* and *d-loop* region (collectively) of *Axis porcinus*.

Statement of conflict of interest

There is no conflict of interest for the contents presented in this paper.

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