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MITOCHONDRIAL DNA MARKER BASED PHYLOGENETIC ANALYSIS OF PAKISTANI NILGAI (*Boselaphus tragocamelus*)

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ABSTRACT

The Nilgai or Blue Bull (*Boselaphus tragocamelus*) is the largest Asian antelope which is endemic to Nepal, Pakistan and Indian regions, where these animals are found in packs. Due to rampant hunting, deforestation and habitat degradation, this vulnerable specie is near threatened in many parts of the world. There is need of conservation efforts both at genomic and geographic level. The present study was designed for genomic characterization of *Boselaphus tragocamelus* in Pakistan by using phylogenetic analysis. Samples from *Boselaphus tragocamelus were* collected from different parks, zoos and natural habitats. DNA was extracted by standard inorganic extraction method. Primer3 software was used for primer designing targeting Mitochondrial specie identification markers such as d-loop, *cytochrome-b* and *cytochrome-c*. After amplification, PCR product was sequenced. Bioinformatics tools were applied for identification of polymorphic loci. Allelic frequency of each locus was calculated. Multidimensional scaling plot illustrated low level of generic diversity among individuals. Phylogenetic analysis revealed conserved neighbouring pattern among different individuals as they shared common ancestry. This is the first report on genomic characterization of Nilgai from Pakistan. The information of selected species of deer is prerequisite for designing effective strategy in future effective practices for conservation. However further genomic investigations should be carried out at a larger scale.

Keywords: Mitochondrial cytochrome-b, cytochrome-c, d-loop, Phylogenetic, Pakistani nilgai.

INTRODUCTION

The largest of the Asian antelopes, the Nilgai (*Boselaphus tragocamelus*) is a bovid that was defined for the first time in 1766 by Pallas. The body of female Nilgai is yellow-brown in color. In males, yellow-brown color steadily changes into blue-grey when they approach the mature age. Their nape and back have erectable mane. A "hair pennant" is visible in the middle of the underside of the neck. White patterns are visible as cheek spots, along edges of the lips and as a throat bib. Underside of body has a thin white stripe which expands in size as it proceeds towards backside. Legs are slim that provide support to stocky body. The body slopes downwards from front towards back. The head is long and slim. Horns of males Blue bull are 20 to 25 cm long. Horns are straight and tilted forward slightly. Favorite habitats of Nilgai are woodland~~s~~ areas and grassy steppe. Abodes of Nilgai in Pakistan are pure desert areas of Cholistan and Thar in Punjab and Sind respectively. Nowadays, this Antelope is naturally found in a narrow strip of land along Indian border in the eastern part of Pakistan. They are not found in herds in this area. Presence of Nilgai in Changa Manga Plantation, near Lahore, has also been reported ~~also~~. According to Sind Wildlife Department, 220 Nilgai were found in Tharparkar district in 1999 (Maan and Chaudhry 2001).

For phylogenetic studies and species identification of different animals, mitochondrial and nuclear DNA sequences have been used frequently in the recent years (Hsieh *et al*., 2001). Molecular phylogenetic and diversity analysis of Pakistani buffalo (Saif *et al.*, 2012; Hussian *et al.*, 2009; 2013a: 2015), goat (Hussain *et al.*, 2013b), sheep (Babar *et al*, 2014; Ahmed*et al.*, 2014), chicken (Babar *et al.*, 2012), camel (Babar *et al.*, 2015) and dog (Tahir *et al.*, 2015) have already been reported but information on wildlife species is inadequate. In this context, present research was planned for genomic characterization by sequencing of mitochondrial *cytochrome-b, cytochrome*-c and *d-loop* regions of Pakistani nilgai (*Boselaphus tragocamelus).*

MATERIALS AND METHODS

Texonomic Specie and Sampling Strategy: Nilgai *(Boselaphus tragocamelus)* specie of animal from *Bovidae* family was selected for this study. Natural habitats of the specie in Pakistan and sampling areas have been depicted in Fig-1. Unrelated animals of the mentioned *Bovidae* family were selected for sampling. A total of 25 fecal and blood samples were collected. The selection was purely based upon phenotypic characteristics. Sampling was done from natural habitats, zoos, parks, wildlife reserves and captive breeding

centers at different places of Pakistan. Sampling details have been provided in Table-1.

Genome Extraction & Purification: Fecal samples were collected using disposable plastic gloves and and stored this mass in 95% ethanol, at room temperature, using a polypropylene bottle. Fecal DNA was extracted as described by Zhang *et al.,* (2006) and quantified by Nanodrop (Thermoscientific, Wilmington USA).

Blood samples were collcted from animals in captivity, in EDTA coated vacutainers and stored at -20 ^{0}C. DNA was extracted by using standard inorganic extraction protocol reported by Maryam *et al*., (2012) and quantified by Nanodrop.

Primer Designing: Reference sequences of complete mitochondrial genome including *cytochrome-b, cytochrome-c* and *d loop* regions for *Boselaphus tragocamelus* (Accession No.NC_020614) was retrieved from NCBI (www.ncbi.nlm.nih.gov). Primers were designed for complete amplification of three loci. Primers were designed using the primer blast of NCBI (www.ncbi.nlm.nih.gov) and synthesized at Genelink, USA.

PCR Amplification and DNA Sequencing: PCR was performed using Bio-Rad Thermocycler. PCR products of all the samples along with 1 kb ladder were run on 1.2 % agarose gel at 100 volts for 35 minutes, to visualize the bands of amplified products. Precipitated PCR products were sequenced using dye labelled dideoxy terminatore sequencing using ABI Genetic Analyzer 3130 XL (Applied Biosystem Inc., Foster city, CA, USA).

Bioinformatic & Statistical Analysis: Alignments of sequences were done with the help of Blast 2 Sequences and Clustal W (Thompson *et al*., 1994). Maximum Parsimony Tree and Bayesian Phylogenetic tree was constructed by using MEGA2 (Kumar *et al.,* 2001). Further analysis was done by using Bioconductor in R (Gentleman *et al.,* 1999) to draw Multidimensional scaling plot and genetic variation plot for all three genes to evaluate the diversity score among individuals.

RESULTS

Phylogenetic analysis of *Boselaphus tragocamelus* using *cytochrome-b* Gene: A total of thirteen polymorphisms were identified in *cytochrome-b* gene of *Boselaphus tragocamelus as* given in Table 2. Allele frequency was calculated. As no heterozygosity was observed so genotypic frequency was found same as allele frequency. Multidimensional scaling (MDS) plot was generated by using 'R'. Plot was figured out by using 1st and 2nd dimensional transformations showing symmetrical variation of genetic distance values in MDS plot. Sequences were analyzed to get evolutionary distance matrix. That matrix was then utilized to plot MDS as shown in Figure 2. Computational model (BH87) was used to generate distance profile for *cytochrome-b* sequence of *Boselaphus tragocamelus*. Genetic variation plot was created by computational model for *Boselaphus tragocamelus* of various regional origins. 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. *Boselaphus tragocamelus* was the target specie. DNA sequences of *cytochrome-b* gene of *Boselaphus tragocamelus* were processed for the phylogenetic analysis. Target specie is encircled in Figure 4.

***Cytochrome-c* Gene based analysis:** A total of sixteen polymorphisms were identified in *cytochrome-c* gene of *Boselaphus tragocamelus as* given in Table 3. No heterozygosity was observed. Allele frequency "1" represents that all samples were monomorphic. MDS plot (figure 5), pair wise evolutionary distance (figure 6) and phylogenetic tree (figure 7) was constructed as described earlier.

Mitochondrial *d-loop* region based analysis: A total of seventeen polymorphisms were identified in mitochondrial *d-loop* region of *Boselaphus tragocamelus* as given in Table 4. No heterozygosty was observed. MDS plot (Figure 8), pair wise evolutionary Distance (Figure 9) and phylogenetic tree (Figure 10) was also contructed.

Mitochondrial *cytochrome-b, cytochrome-c* and *d-loop* region (three genes combined sequence) based analysis: Sequence of three genes (*cytochrome-b, cytochrome-c* and *d-loop*) were combined and 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 (Figure 11). pair wise Evolutionary distance (Table 5) and estimates of evolutionary divergence between sequences (Figure 12) were calculated. Phylogenetic tree was constructed by using maximum likelihood method as described earlier. DNA sequences of *cytochrome-b, cytochrome-c and d-loop* region (combined sequence) of animals under study were routed for phylogenetic analysis (Figure 13).

Table 1. Sampling details of *Boselaphus tragocamelus*[a] samples used in this study.

Sr. No.	Samples	Source	Google Coordinates
1	BT[a]5 BT17	Bahawalpur Zoo	29°24'8.7"N 71°40'54.5"E
2	BT7, BT23	Bahria Town Lahore	31°18'51.5"N 74°12'11.7"E
3	BT10, BT19, BT20	Bahria Town Rawalpindi	33°29'45.2"N 73°6'20.3"E
4	BT8	Basti Bahadurpur Multan	30°15'27.9"N 71°29'48.2"E
5	BT2	Changa Manga Kasur	31°5'19.3"N 73°57'44.7"E
6	BT4	Charagh Abad, T T Sing	31°20'6.3"N 72°46'2.4"E
7	BT15 BT22	Gatwala Wildlife Breeding Centre, Faisalabad	31°28'42.7"N 73°12'36.7"E
8	BT16	Lahore safari park, Lahore	31°22'53.9"N 74°12'41.6"E
9	BT18	Lahore Zoo, Lahore	31°33'22.7"N 74°19'34.0"E
10	BT9, BT11	Lal Suhanra National Park Bahawalpur	29°19'1.4"N 71°54'16.4"E
11	BT14, BT21	Lohi Bher Wildlife Park Rawalpindi	33°57'49.5"N 73°11'93.1"E
12	BT24	Indo-Pak border, Bahawalnagar	29°59'57.1"N 73°15'31.8"E
13	BT25 BT3	Peerowal Khanewal	30°20'22.7"N 72°2'2.4"E
14	BT1 BT6	Head Balloke Raavi River	31°11'25.9"N 73°52'32.6"E
15	BT13	Vehari Wildlife Park Vehari	30°2'14.7"N 72°21'2.6"E
16	BT12	Wildlife Park Kamalia, T T Sing	30°42'52.9"N 72°40'25.9"E

Table 2. Polymorphisms in *cytochrome-b* gene of *Boselaphus tragocamelus.*

No.	Base Position[a]	*Change in Nucleotide (Wild to Mutant)*	Allele Frequency	
			A	B
1	14180	A→ C	0	1[b]
2	15286	C→ T	0	1 [b]
3	15302	T→ G	0	1 [b]
4	14293	C→T	0.92	0.08
5	14635	C→T	0.64	0.36
6	15121	T→C	0.92	0.08
7	15134	C→T	0.92	0.08
8	15135	T→C	0.92	0.08
9	15138	G→A	0.92	0.08
10	15154	C→T	0.64	0.36
11	15179	G→A	0.80	0.20
12	15252	A→G	0.84	0.16
13	15290	T→C	0.60	0.40

a. with reference to NC_020614
b. all samples were monomorphic

Table 3. Polymorphisms in *cytochrome-c* gene of *Boselaphus tragocamelus.*

No.	Base Position[a]	*Change in Nucleotide (Wild to Mutant)*	Allele Frequency	
			A	B
1	5497	T→ A	0	1
2	6260	G→ A	0	1
3	6395	G→ A	0	1
4	6668	G→ A	0	1
5	6707	C→ T	0	1
6	5469	A→G	0.80	0.20
7	5472	C→T	0.80	0.20
8	5493	A→G	0.80	0.20
9	5496	G→A	0.80	0.20
10	5867	C→T	0.64	0.36
11	5871	A→G	0.64	0.36
12	5937	T→C	0.64	0.36

13	6260	G→A	0.88	0.12
14	6395	G→A	0.88	0.12
15	6668	G→A	0.88	0.12
16	6818	C→T	0.68	0.32

a. with reference to NC_020614

Table 4. Polymorphisms in *d-loop* region of *Boselaphus tragocamelus.*

No.	Base Position[a]	*Change in Nucleotide (Wild to Mutant)*	Allele Frequency	
			A	B
1	15684	T→ C	0	1
2	15834	C→ T	0	1
3	16284	C→ G	0	1
4	15556	G→A	0.68	0.32
5	15606	T→C	0.76	0.24
6	15647	A→G	0.88	0.12
7	15662	T→C	0.88	0.12
8	15663	C→T	0.88	0.12
9	15685	C→T	0.64	0.36
10	15705	T→C	0.88	0.12
11	15713	G→A	0.88	0.12
12	15835	T→C	0.64	0.36
13	16085	T→C	0.88	0.12
14	16241	A→G	0.80	0.20
15	16283	A→T	0.80	0.20
16	16285	C→G	0.64	0.36
17	16288	A→G	0.80	0.20

a. with reference to NC_020614

Table 5. *Cytochrome-b, cytochrome-c and d-loop* region based evolutionary analysis of *Boselaphus tragocamelus.*

[	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
[1]																									
[2]	0.00365																								
[3]	0.00169	0.00253																							
[4]	0.00478	0.00281	0.00365																						
[5]	0.00309	0.00169	0.00197	0.00337																					
[6]	0.00197	0.00281	0.00084	0.00393	0.00225																				
[7]	0.00112	0.00478	0.00281	0.0059	0.00422	0.00309																			
[8]	0.0045	0.00309	0.00393	0.0059	0.00309	0.00365	0.00393																		
[9]	0.00422	0.0045	0.00253	0.00562	0.00393	0.00281	0.00422	0.00478																	
[10]	0.00393	0.0059	0.00506	0.00534	0.00478	0.00534	0.00393	0.00618	0.00253																
[11]	0.00309	0.00169	0.00197	0.00337	0	0.00225	0.00422	0.00309	0.00393	0.00478															
[12]	0.00253	0.00393	0.00141	0.00506	0.00337	0.00112	0.00197	0.00309	0.00281	0.00534	0.00337														
[13]	0.0045	0.00422	0.00337	0.00197	0.00309	0.00365	0.00393	0.00393	0.00422	0.0045	0.00309	0.00309													
[14]	0.00478	0.00393	0.00422	0.00675	0.00337	0.00337	0.00478	0.00365	0.00169	0.00365	0.00337	0.00337	0.00534												
[15]	0.00365	0.00056	0.00309	0.00337	0.00225	0.00281	0.00422	0.00309	0.00506	0.00646	0.00225	0.00393	0.00478	0.00393											
[16]	0.00253	0.00393	0.00141	0.00506	0.00337	0.00169	0.00365	0.00253	0.00393	0.00646	0.00337	0.00225	0.00478	0.00506	0.00393										
[17]	0.00141	0.00225	0.00309	0.00337	0.00393	0.00281	0.00253	0.00478	0.00506	0.00422	0.00393	0.00393	0.00478	0.00562	0.00225	0.00393									
[18]	0.00253	0.00112	0.00141	0.00393	0.00056	0.00169	0.00365	0.00253	0.00337	0.00534	0.00056	0.00281	0.00365	0.00281	0.00169	0.00281	0.00337								
[19]	0.0045	0.00084	0.00337	0.00197	0.00141	0.00365	0.00562	0.00393	0.00534	0.00506	0.00141	0.00478	0.00393	0.00478	0.00141	0.00478	0.00309	0.00197							
[20]	0.00197	0.00225	0.00028	0.00337	0.00169	0.00056	0.00309	0.00365	0.00225	0.00478	0.00169	0.00169	0.00309	0.00393	0.00281	0.00169	0.00281	0.00112	0.00309						
[21]	0.00365	0.00393	0.00253	0.00112	0.00225	0.00281	0.00478	0.00534	0.0045	0.00478	0.00225	0.00393	0.00141	0.00562	0.0045	0.00393	0.0045	0.00281	0.00309	0.00225					
[22]	0.00365	0.00281	0.00253	0.0045	0.00112	0.00337	0.00309	0.00253	0.00393	0.00478	0.00112	0.00225	0.00253	0.00337	0.00337	0.00393	0.00506	0.00169	0.00253	0.00281	0.00337				
[23]	0.00309	0.00337	0.00197	0.0045	0.00337	0.00112	0.00253	0.00253	0.00281	0.00478	0.00337	0.00056	0.00253	0.00337	0.00337	0.00281	0.00337	0.00281	0.00422	0.00169	0.00393	0.00281			
[24]	0.00112	0.00422	0.00225	0.00365	0.00253	0.00309	0.00225	0.00562	0.00478	0.00281	0.00253	0.00365	0.00393	0.0059	0.00478	0.00365	0.00253	0.00309	0.00337	0.00253	0.00253	0.00309	0.00422		
[25]	0.16554	0.16582	0.1647	0.16554	0.1661	0.1647	0.16554	0.16695	0.1661	0.16835	0.1661	0.16498	0.16554	0.16695	0.16526	0.16554	0.16582	0.16554	0.16667	0.1647	0.16526	0.16639	0.16526	0.16667	
[26]	0.00225	0.00309	0.00169	0.00422	0.00253	0.00141	0.00225	0.00281	0.00253	0.0045	0.00253	0.00141	0.00281	0.00309	0.00309	0.00253	0.00309	0.00197	0.00393	0.00141	0.00309	0.00253	0.00141	0.00337	0.1647

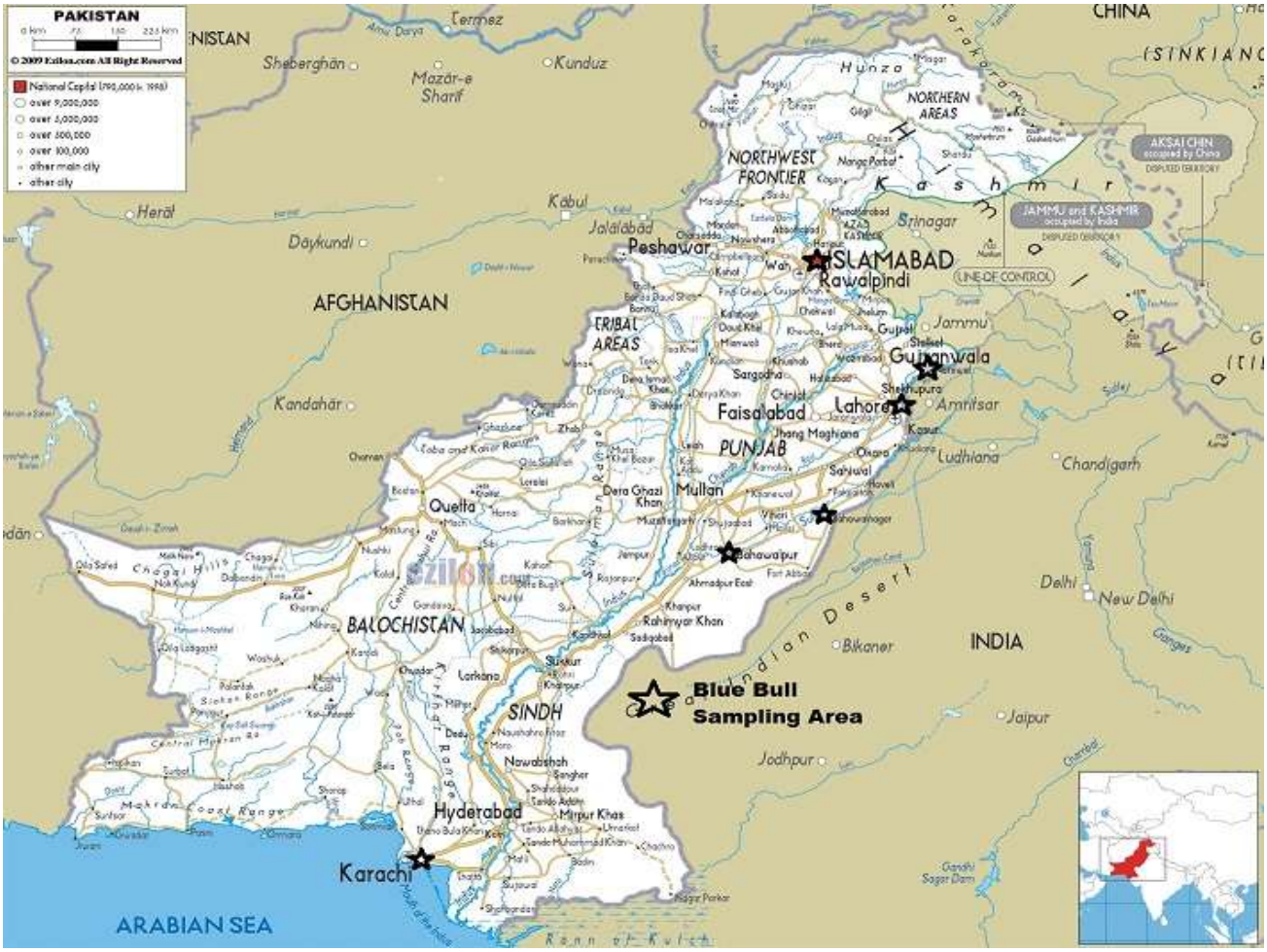

Figure 1. Map of Pakistan showing selected areas for sampling of *Boselaphus tragocamelus* (Blue bull). The fig was modified from http://www.ezilon.com (20-4-2015).

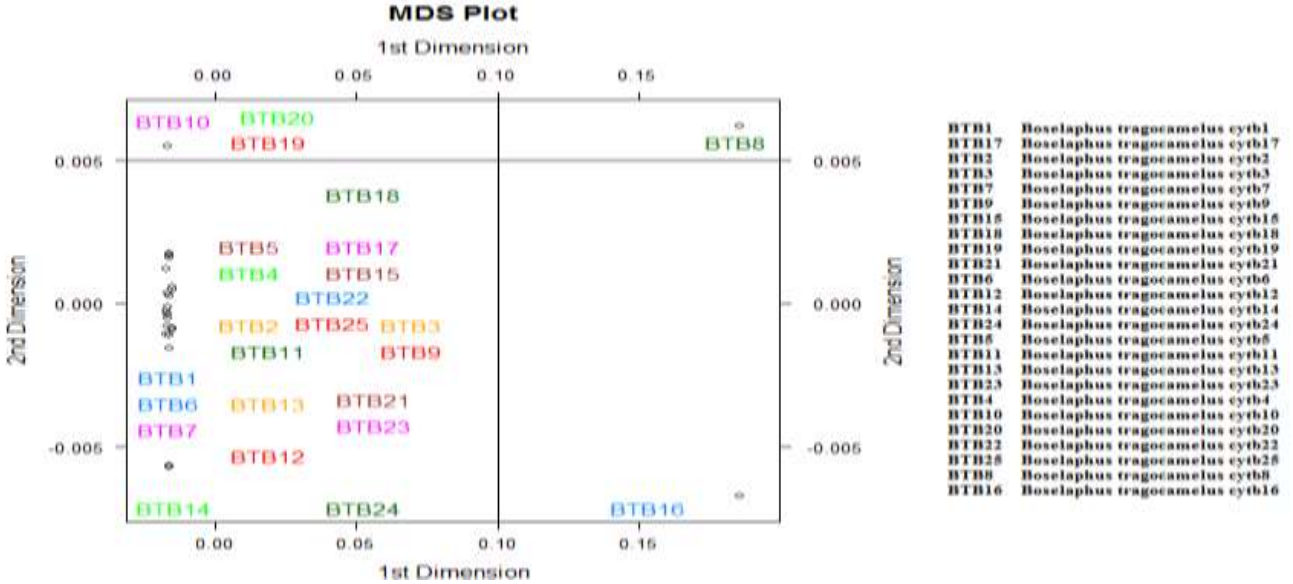

Figure 2. Multidimensional scaling plot of mitochondrial genomic region, *cytochrome-b* for *Boselaphus tragocamelus.*

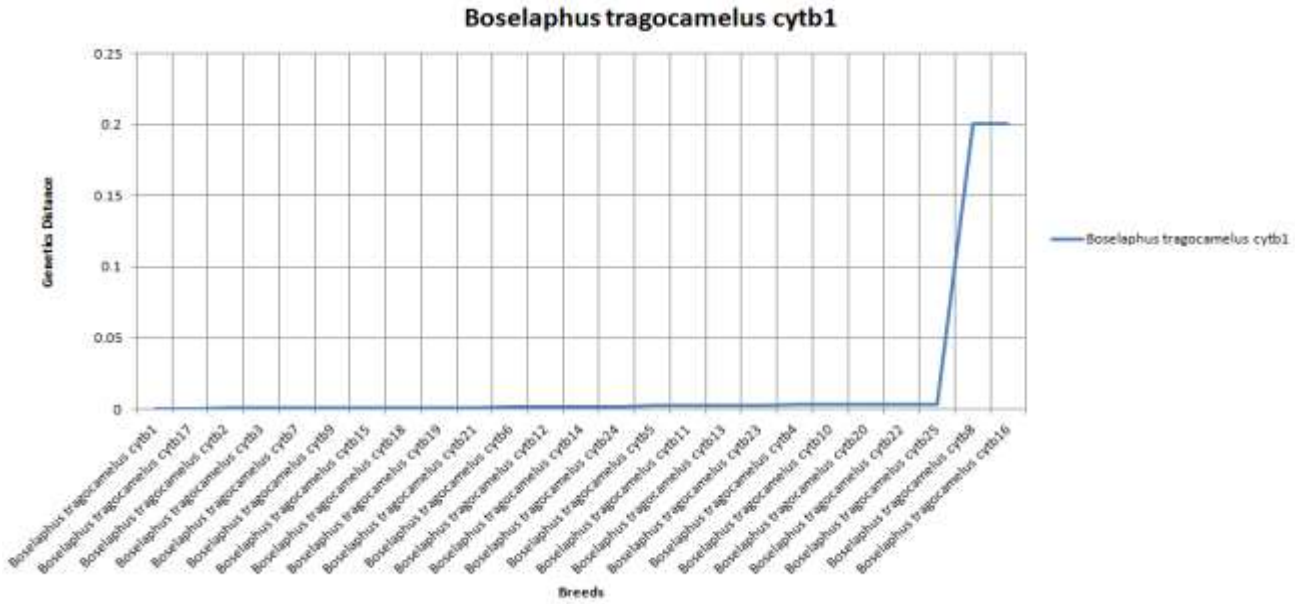

Figure 3. *Cytochrome-b* based Genetic variation plot of *Boselaphus tragocamelus.*

Figure 4. Phylogenetic Neighbor joining tree (Circular) of *cytochrome-b* gene of *Boselaphus tragocamelus.*

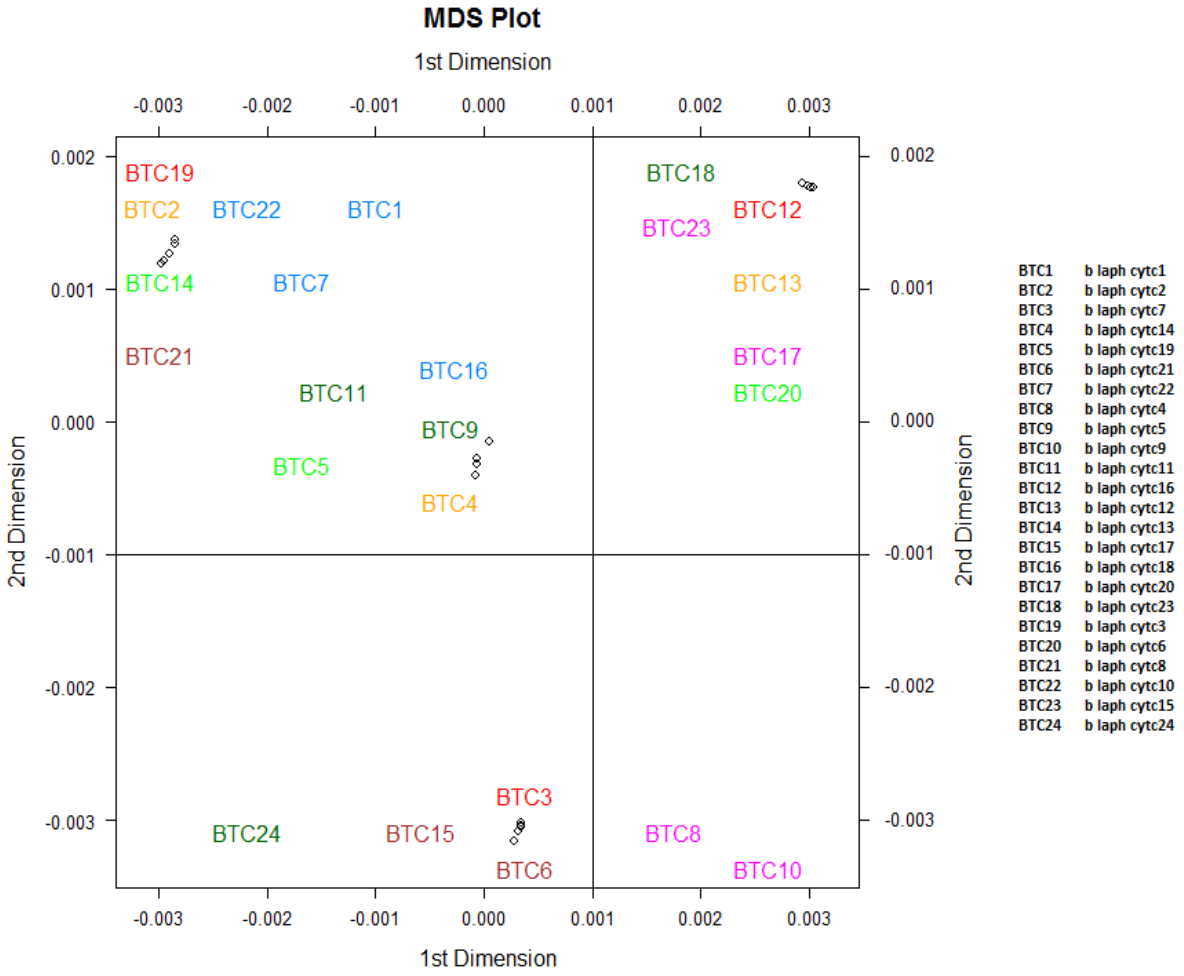

Figure 5. Multidimensional scaling plot of mitochondrial genomic gene, *cytochrome-c* for *Boselaphus tragocamelus.*

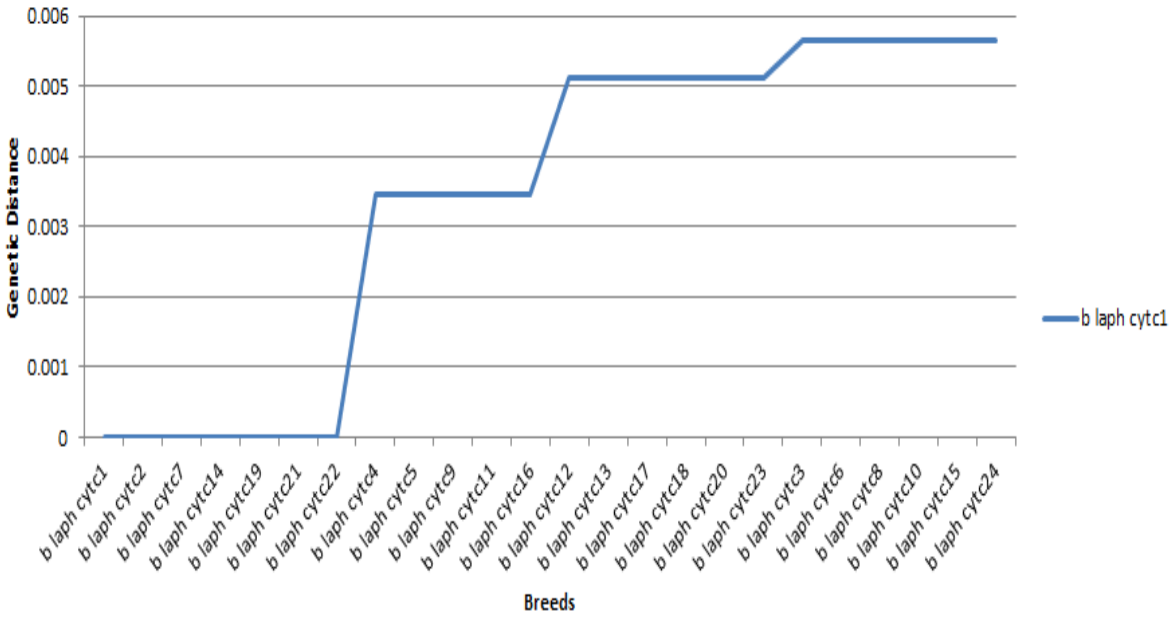

Figure 6. *Cytochrome-c* based genetic variation plot of *Boselaphus tragocamelus.*

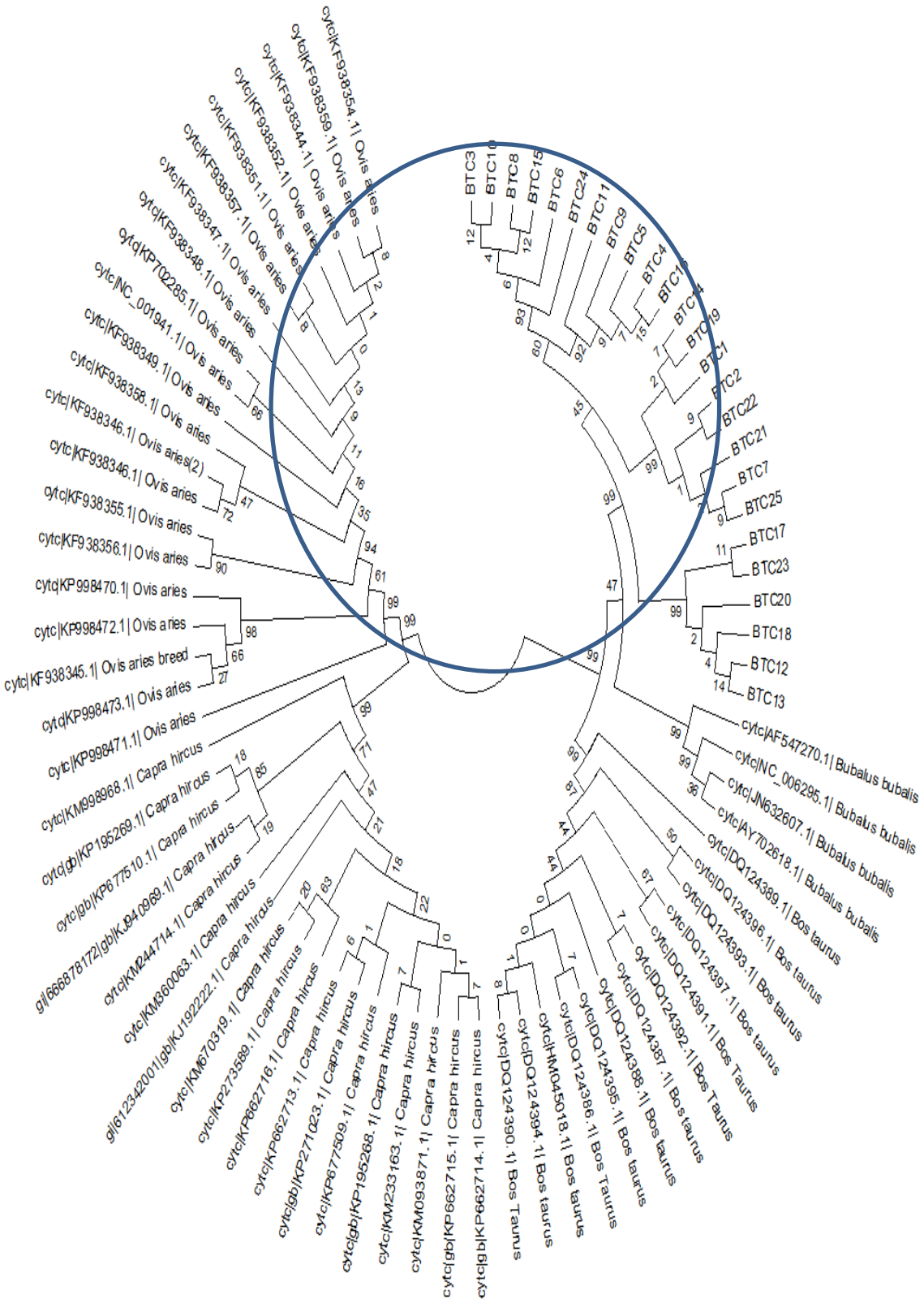

Figure 7. Phylogenetic tree (Circular) of *cytochrome-c* gene of *Boselaphus tragocamelus.*

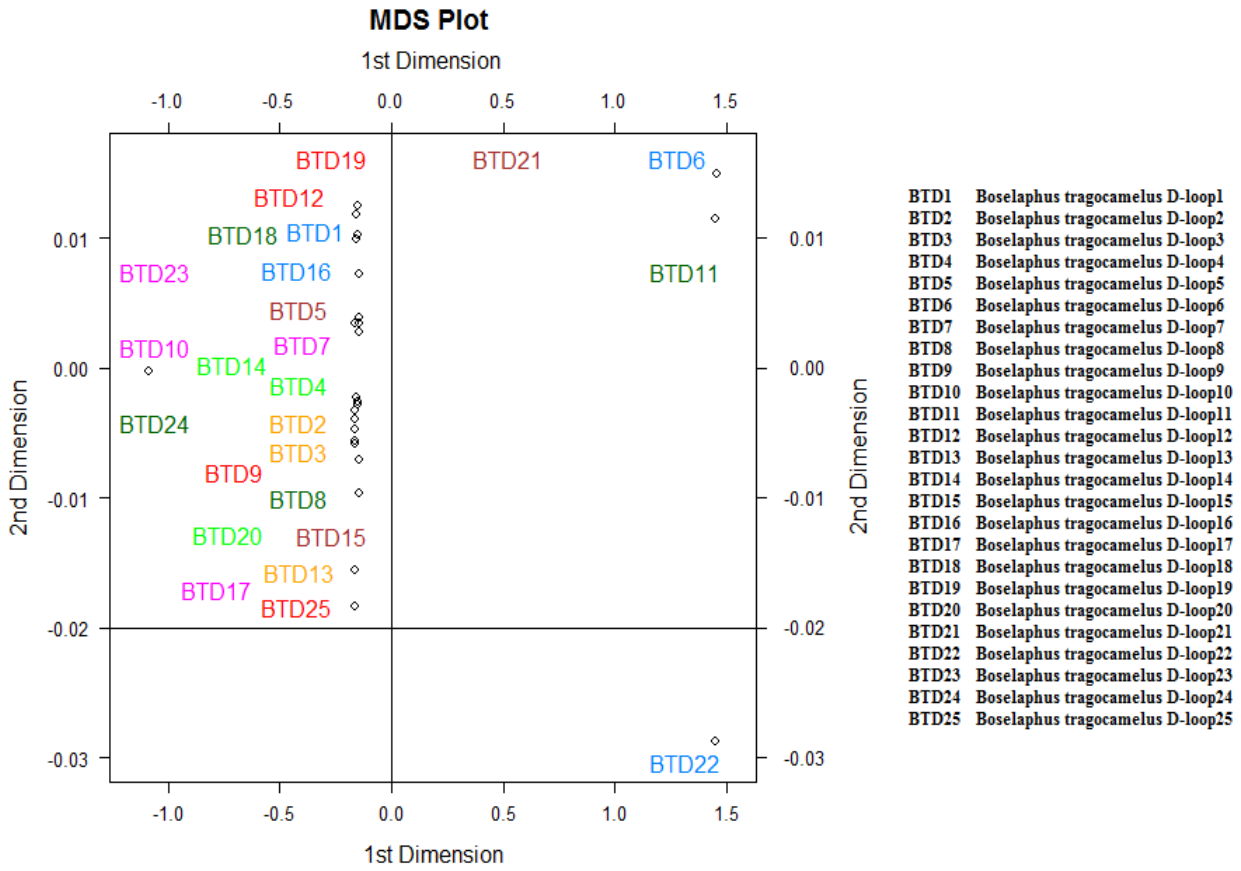

Figure 8. Multidimensional scaling plot of mitochondrial genomic *d-loop region* for *Boselaphus tragocamelus.*

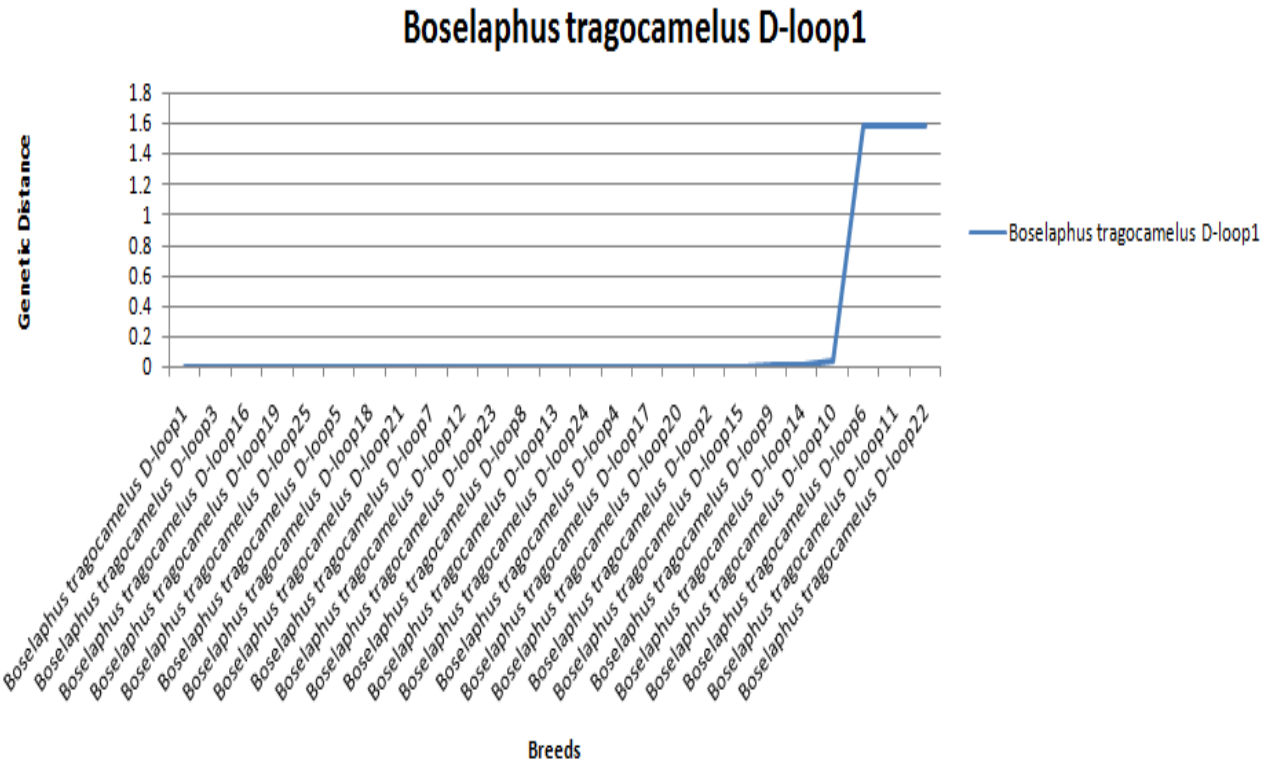

Figure 9. *d-loop* sequence based Genetic variation plot of *Boselaphus tragocamelus.*

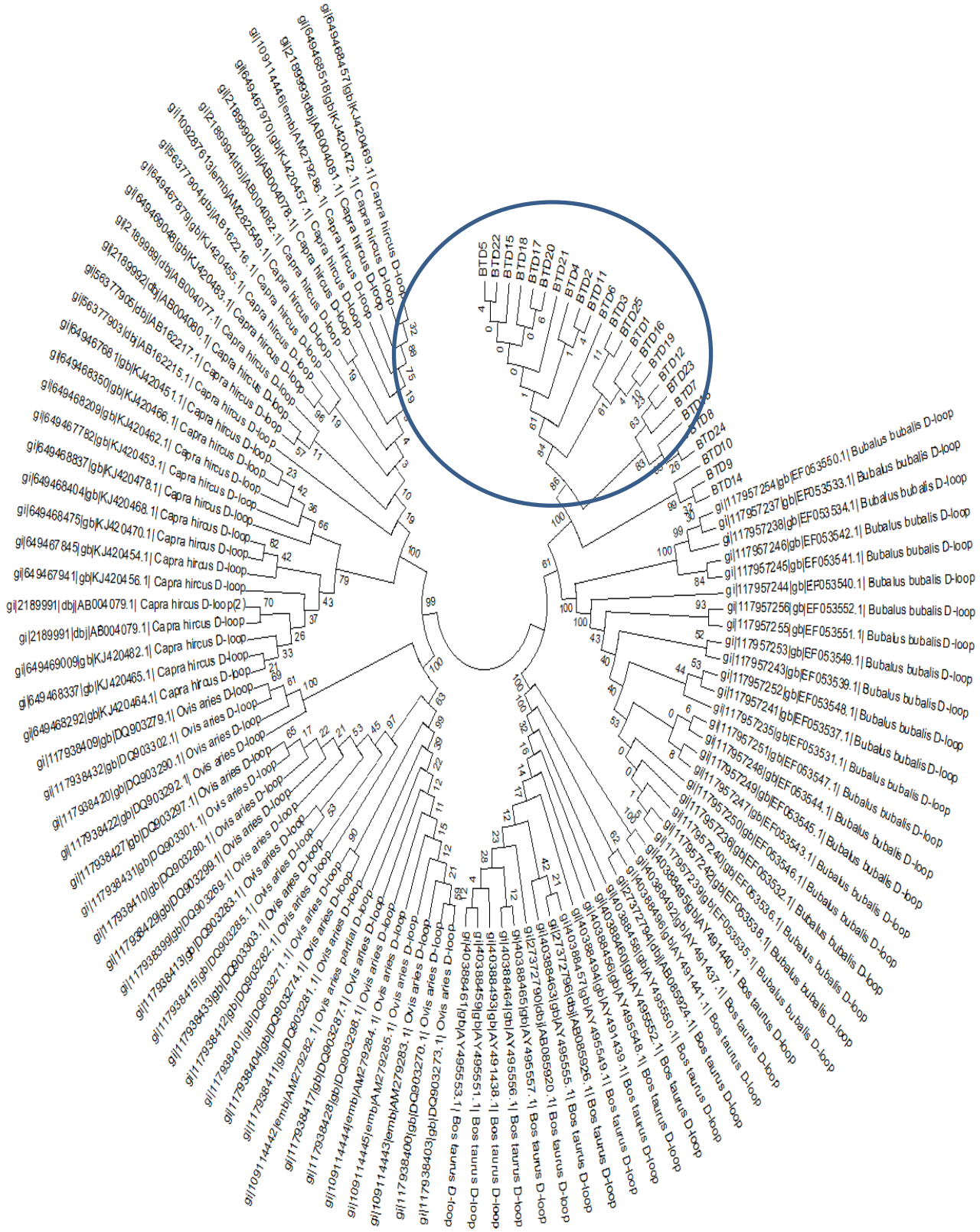

Figure 10. Phylogenetic tree (Circular) of *d-loop* region of *Boselaphus tragocamelus.*

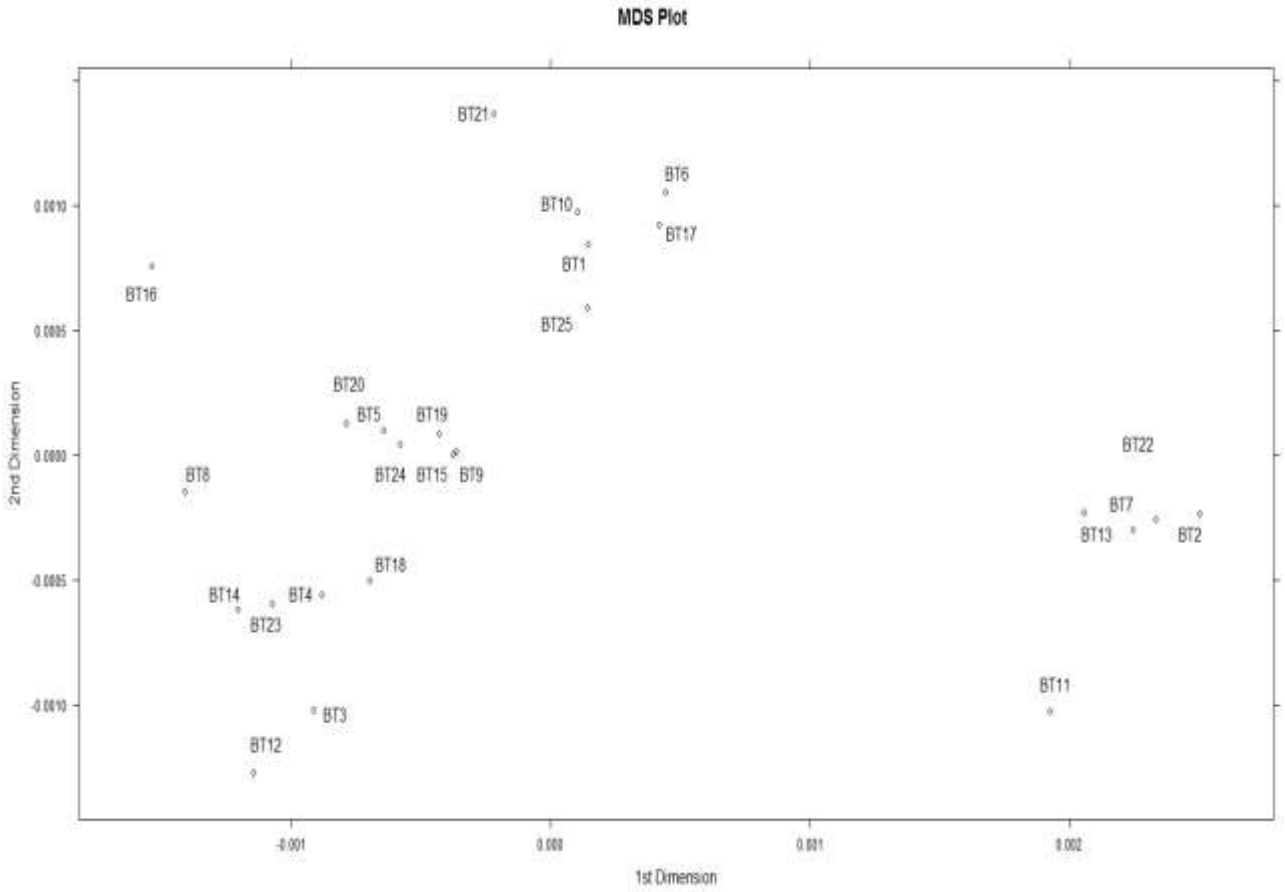

Figure 11. Multidimensional scaling plot of mitochondrial genomic *cytochrome-b, cytochrome-c and d-loop region* for *Boselaphus tragocamelus.*

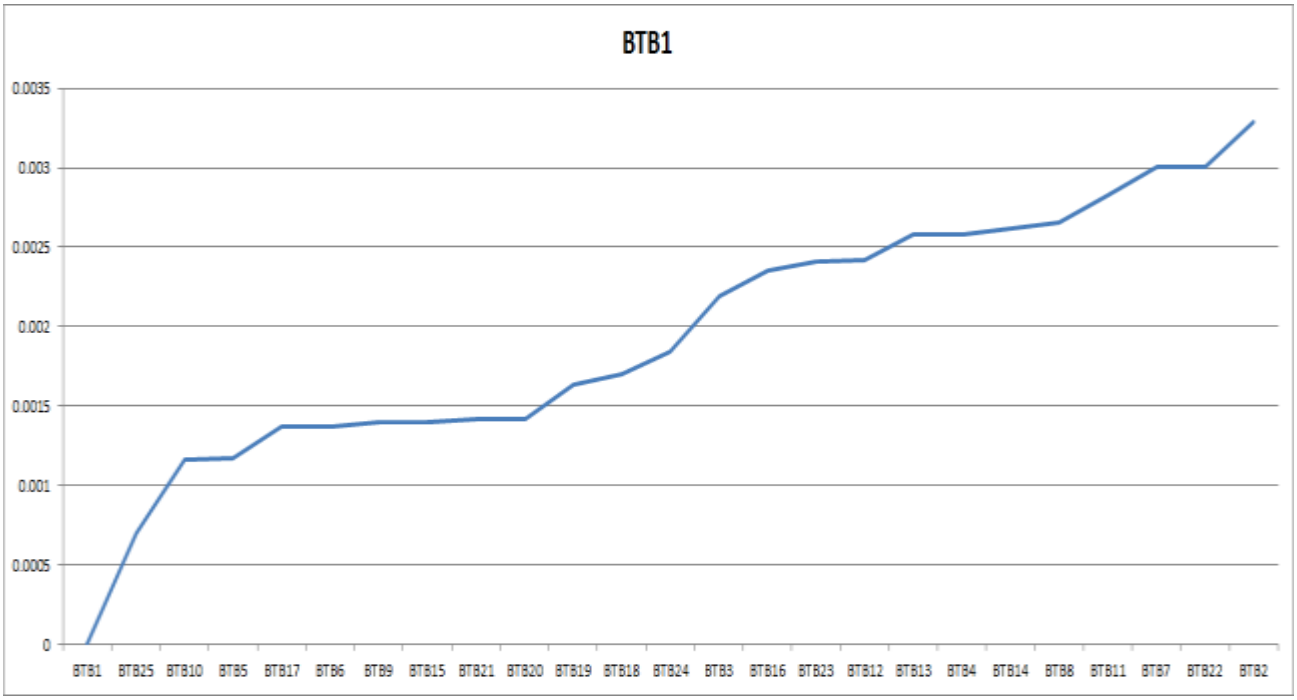

Figure 12. *Cytochrome-b, cytochrome-c* and *d-loop* region based genetic variation plot of *Boselaphus tragocamelus.*

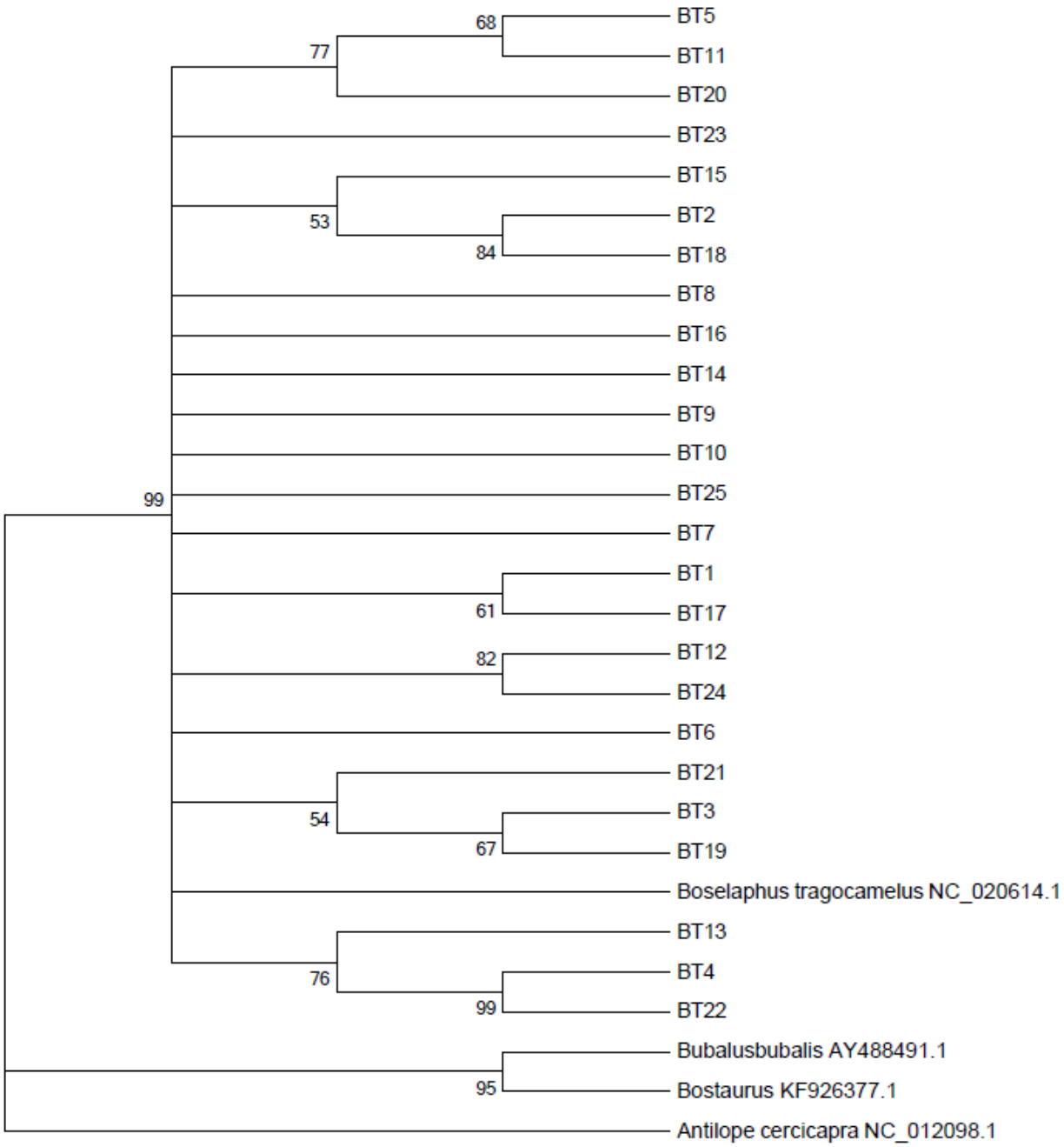

Figure 13. Phylogenetic tree (Rectangular) of *Cytochrome-b, cytochrome-c* and *d-loop* region of *Boselaphus tragocamelus.*

DISCUSSION

Conservation of genome sequences might play a significant role in species survival and ecosystem conservation in the future. For the purpose of collecting genetic information ~~for~~ on *Boselaphus tragocamelus* specie in Pakistan, mitochondrial *cytochrome-b, cytochrome-c and d-loop* regions were analysed to study the genetic diversity and used for construction of Phylogenetic tree (Giovambattista *et al.*, 2001; Feral *et a.l*, 2006 and Mwacharo *et a1*., 2006). DNA sequence based techniques are helpful for evaluating genetic changeability between different populations (Haig; 1998). The wild animal's conservation is rare and depends on protection of genetic differences which is sign of difference in genomes (Crozier,1992; Lynch and Milligan, 1994). To assess the level of genetic variation,

an 1139 bp fragment, in the *cytochrome-b* gene of the mitochondrial DNA, from 25 deer individuals was PCR-amplified and sequenced and analysed. A total of thirteen variable sites were observed in *cytochrome-b* gene of *Boselaphus tragocamelus*. Out of these, three variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 10 transitions and three transversions. 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 the same. The average heterozygosity values for endangered and non-endangered populations are often lower and higher values, respectively (Frankham *et al*., 2002). So, our results illustrate distribution of specie inclined more towards endangered, which is alarming and demands immediate measures for its conservation. In some other related studies as well, overall genotypic frequency has been found to be homozygous as Nielsen *et al*., (2008), Qureshi *et al*., (2004) and Dellicour *et al.,* (2011).

Pepin *et al*. (1995) reported that conservation of genome nucleotide sequence occurs in Nilgai *(Boselaphus tragocamelus).* Gallagher *et al*. (1998) established that Nilgai was karyotypically derivative to genus of Bovinae; various resultant chromosomal conditions familiar to *B. tragocamelus*, the buffalo from Africa and various Tragelaphini can be convergent. Nucleotide sequence of different genes such as *interferon2*, *Toll like receptor3* and prion protein suggest lineage share of this species with buffalo, kudu and cattle (Das *et al*. 2006: Dhara *et al*. 2007: Seabury *et al*., 2004). Similar outcomes have been illustrated in the phylogenetic analysis (Fig-13). Genetic variation information is prerequisite for future conservation strategy (Crandall *et al*., 2000). It is recommended that further genomic investigations should be carried out at a larger scale.

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