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Research Article

Mitochondrial *Cytochrome-b, Cytochrome-c* and *d-loop* Region Based Phylogenetic and Diversity Analysis in Blackbuck *(Antilope cervicapra)*

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Abstract

The present study was designed to find diversity analysis of *Antilope cervicapra* family in Pakistan. Fecal samples of *Antilope cervicapra* were collected from their different habitats of Pakistan. Fecal DNA was extracted and Polymerase Chain Reaction (PCR) was performed. Sequencing was performed by Big DyeTM Terminator method. Diversity and phylogenetic analysis was performed by different Bioinformatics tools. Less genetic variability was observed within *Antilope cervicapra* population through Multi-Dimensional Scaling (MDS). However, significant genetic variation was observed among other species and *Antilope cervicapra*. Phylogenetic analysis revealed distinct clade of this specie with respect to other species of deer. This is the first report from Pakistan that could help for designing effective strategy in future conservation practices of deer species.

Keywords: *Blackbuck, Phylogeny, Mitchondrial, Cytochrome, Phylogenetic diversity*

Kara Antilop'ta *(Antilope cervicapra)* Mitokondriyal *Sitokrom-b, Sitokrom-c* ve *d-loop* Bölgelerine Dayalı Filogenetik ve Çeşitlilik Analizi

Öz

Bu çalışma Pakistan'daki *Antilope cervicapra* ailesinin çeşitlilik analizini belirlemek için tasarlandı. *Antilope cervicapra* dışkı örnekleri Pakistan'ın farklı habitatlarından toplandı. Fekal DNA ekstrakte edildi ve Polimeraz Zincir Reaksiyonu (PCR) yapıldı. Dizileme işlemi Big DyeTM Terminator metodu ile yapıldı. Çeşitlilik ve filogenetik analizlerde farklı biyoenformatik araçlar kullanıldı. *Antilope cervicapra* popülasyonunda Çok Boyutlu Ölçeklendirme (MDS) değerlendirmesinde düşük düzeyde genetik değişkenlik gözlendi. Bununla birlikte, diğer türler ve *Antilope cervicapra* arasında önemli genetik varyasyon belirlendi. Filogenetik analiz, bu türün diğer geyik türlerine göre belirgin bir tür olduğunu ortaya koydu. Bu araştırma, Pakistan'dan geyik türlerinin gelecekteki koruma uygulamalarında etkili bir strateji tasarlanmasına yardımcı olabilecek ilk rapordur.

Anahtar sözcükler: *Kara antilop, Filogenetik, Mitkondrial, Sitokrom, Filogenetik çeşitlilik*

INTRODUCTION

It is necessary to keep the information of wild animal genetic diversity for the better management and conservation of these wild species [1]. So, present day biological studies are needed to be focused on conservation of genetic diversity. The assessment of the genetic diversity is needed for maintenance of sustainable hunting, conservation and improvement of genetic resources of animals of a specific population [2].

Male and female Blackbucks *(Antilope cervicapra)* are somewhat morphologically different from each other. Females are yellow in color and most of them do not have horns. Female blackbuck has thin and slim body. Whereas, males have horns and horns grow before the change of their body color. Blackbucks can breed throughout the year, but March to May and August to October are top breeding periods.

Blackbucks originally belong to Indian subcontinent. They were found everywhere in this area from plains up to

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mountains. Nowadays, human activities have destructed their habitat so they are found only in isolated places. In our country of Pakistan, blackbucks are found in Cholistan and Thar area of Punjab and Sindh along eastern border areas. At present, there is no specific area for their living in Pakistan. Now they are found as captive in different places like Kirthar and Lal Suhanra National Parks. The antilope specie is now extinct in Sindh. However, a large population of the species exits in Khairpur's Mehrano reserve. Some other places, where blackbuck are found in Pakistan are zoos and different wildlife centers. In 2008; more than 1500 Blackbucks were found in different breeding centers of Punjab and Sindh in Pakistan. DNA barcoding has been used to find genetic diversity at species level. It is considered as standardized approach to show interspecific variations from the mitochondrial region [3].

For phylogenetic studies and species identification of different animals, *Cytochrome-b* along with other mitochondrial DNA markers has been used frequently in the recent times [4]. So, the current study was designed to identify the polymorphisms in *Antilope cervicapra* phylogenetic relationships within and among other animals.

MATERIALS and METHODS

Taxonomic Species and Sampling Strategy

Blackbucks *(Antilope cervicapra)* species were selected from their natural habitats, parks, zoos, and captive breeding centers at various places of Pakistan. Sampling areas are shown in *Table 1*. Selection was carried out on the basis of phenotypic characteristics. Fecal samples (n=25) were collected from species. Permission was taken from the competent authorities of sample collection areas.

Genome Extraction and Purification

Fecal samples were taken and preserved in 95% ethanol at room temperature. DNA extraction from fecal mass was extracted through inorganic method by the protocol of Zhang et al.[5] and with minor modifications as by Maryam et al.[6]. Briefly, 1.0-1.5 g of feces material was taken and centrifuged with 5 mL ethanol (4000 × *g*, 2 min). The washing step was repeated once using 5 mL TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Three mL TNE buffer (10 m mol/L Tris-Cl, 0.5% SDS, 1 m mol/L $CaCl_2$) and 50 µL Proteinase K (20 mg/mL) were added to the centrifuge tube, and the whole was incubated at 55°C for 1-2 h. The lysate was centrifuged (4000 × *g*, 1 min) to pellet the fecal particle. The supernatant was centrifuged again with potato starch and pipetted into a new 2 mL centrifuge tube, to which 150 µL NaCl solution (3.5 mol/L) and 250 µL CTAB solution (0.7 M NaCl, 10% cetyl trimethyl-ammonium bromide, CTAB) were added, followed by incubation at 70°C for 10 min. The mixture was extracted twice using an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). Elute was filtered and preserved with 200 µL TE, and 50 µg/mL RNase was added at 4°C. DNA quantity was measured by Nanodrop (Thermoscientific, Wilmington USA).

Table 1. *Sampling details of Antilope cervicapra samples*

Sr. #	Samples ID	Source	Google Coordinates	Number of Samples
1	AC18, AC19	Bahawalpur Zoo, Bahawalpur	29°24'8.7"N 71°40'54.5"E	2
2	AC13, AC21	Bahria Town Lahore	31°18'51.5"N 74°12'11.7"E	2
3	AC20, AC12	Bahria Town Rawalpindi	33°29'45.2"N 73°6'20.3"E	2
4	AC16	Basti Bahadurpur Multan	30°15'27.9"N 71°29'48.2"E	1
5	AC9	Changa Manga, Kasur	31°5'19.3"N 73°57'44.7"E	1
6	AC24	Charagh Abad, T T Sing	31°20'6.3"N 72°46'2.4"E	1
7	AC2	Peerowal Khanewal	30°20'22.7"N 72°2'2.4"E	1
8	AC25	Khangur Ghotki, Sindh	27°52'10.8"N 69°25'2.5"E	1
9	AC6, AC10	Kirthar National Park, Sindh	25°41'27.8"N 67°31'23.4"E	2
10	AC3	Lahore Safari Park	31°22'53.9"N 74°12'41.6"E	1
11	AC5	Lahore Zoo, Lahore	31°33'22.7"N 74°19'34.0"E	1
12	AC15, AC11	Lal Suhanra National Park	29°19'1.4"N 71°54'16.4"E	2
13	AC4	Lohi Bher Wildlife Park Rawalpindi	33°57'49.5"N 73°11'93.1"E	1
14	AC17	Mir of Khairpurs Mehrano Reserve, Sindh	27°16′47.1″N 68°40′32.6″E	1
15	AC7	New Jatoi, Nawab Shah, Sindh	26°47′37.6″N 67° 59′28.0″E	1
16	AC1, AC23	Rajoa Saadat, Chiniot	31°38′35.5″N 72°58′26.7″E	2
17	AC22, AC14	Head Balloke Raavi River	31°11'25.9"N 73°52'32.6"E	2
18	AC8	Wildlife Farms Raiwind Road Lahore	31°23'5.5"N 74°14'8.5"E	1
TOTAL				25

Primer Designing

Amplification of complete mitochondrial genome including three loci, *cytochrome-b, Cytochrome-c* and *d loop* regions of *Antilope cervicapra* was carried out. Reference sequences (Accession No.NC_020614) were taken from NCBI for primer designing and primers were designed by the primer blast of NCBI.

PCR and DNA Sequencing

PCR amplification and DNA sequencing was performed as described by the Abbas et al.[7] and Nadeem et al.[8]. PCR was carried out in four steps. Initial denaturing was performed at 95°C for 2 min in first step. Second step comprised of 30 cycles each for 30 s at 95°C for denaturation, 30 s at 51°C-54°C (as optimized per locus) for primer annealing and 2 min at 72°C for extension. Third step was final extension for 10 min at 72°C and Fourth step was maintaining the final temperature at 4°C until completion of PCR. PCR products along with 1 kb ladder were run on 1.2% agarose gel at 100 volts for 35 min. to visualize the bands of amplified products.

DNA Sequencing and Sequence Submission to NCBI

Ethanol was used for precipitation of PCR products. 40 mL of 75% ethanol was added to each 10 mL reaction to final concentration of 60%. The reaction mixtures were mixed left at room temperature for 20 min. Then, these were centrifuged at 16000 x g (14000 rpm for 20 min) at 4°C. The supernatant was discarded and pellets were washed with 100 mL of 70% ethanol. Then pellets were dissolved in 15 mL of deionized and were sequenced by dideoxy terminator sequencing by using ABI Genetic Analyzer 3130 XL (Applied Biosystem Inc., Foster city, CA, USA). The sequences (41) were submitted to GeneBank (NCBI) with accession numbers MH155269, MH181808-181822; MK051009-MK051020; MH920321- MH920333.

Bioinformatics and Statistical Analysis

Sequenced were aligned through Alignments Blast 2 and Clustal W tools [9]. Phylogenetic analysis was carried out using the MEGA2 [10]. Bayesian Phylogenetic tree and Maximum Parsimony Tree were used. Diversity score was evaluated by the R statistical package [11]. Multidimensional scaling and genetic variation plots were drawn for all three genes.

RESULTS

In *Antilope cervicapra*, 1139 bp fragment sequence of mitochondrial *Cytochrome-b* gene was analysed. A total of thirteen variable sites were observed *(Table 2)*. Out of these, 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 11 transitions and two transversions as shown in *Table 2*. Low frequency of mutant allele and no heterozygous individuals was observed.

Table 2. *Polymorphisms identified in cytochrome-b gene of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	14174	C→A	0	1
2	14177	A→C	0	1
3	14256	G→A	0	1
4	14544	A→G	0	1
5	15220	G→A	0	1
6	15293	G→A	0	1
7	13650	G→A	0.56	0.44
8	13657	C→T	0.52	0.48
9	13660	A→G	0.80	0.20
10	13664	A→G	0.56	0.44
11	14532	T→C	0.76	0.24
12	14710	A→G	0.80	0.20
13	14735	T→C	0.80	0.20

[a] *with reference to NC_012098*

Table 3. *Polymorphisms identified in cytochrome-c gene of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	5890	C→T	0	1
2	6577	A→G	0	1
3	5420	A→G	0.96	0.04
4	5459	G→A	0.92	0.08
5	5510	G→A	0.96	0.04
6	5536	C→T	0.88	0.12
7	5579	C→T	0.96	0.04
8	5616	T→C	0.92	0.08
9	5665	C→T	0.84	0.16
10	5811	T→C	0.96	0.04
11	5990	A→G	0.96	0.04
12	6032	G→A	0.80	0.20

[a] *with reference to NC_012098*

In the *Cytochrome-c* gene of the mitochondrial DNA, 1544 bp fragment from *Antilope cervicapra* individuals was analysed. A total of twelve variable sites were observed *(Table 3)*. Out of these, three variations were found monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. There were 12 transitions but no 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.

Mitochondrial *d-loop* region (997 bp fragment) of *Antilope cervicapra* individuals was sequenced and analysed. A total of sixteen variable sites were observed as shown in *Table 4*. Out of these, ten variations were monomorphic for mutant allele. Remaining animals were also homozygous both for wild and mutant allele. The variable sites were comprised of 16 transitions and no transversion. No heterozygous individuals were found.

Table 4. *Polymorphisms identified in mitochondrial d-loop region of Antilope cervicapra*

No.	Base Position[a]	Change in Nucleotide (Wild to Mutant)	Allele Frequencies	
			A	B
1	15578	A→G	0	1
2	15591	G→A	0	1
3	15644	A→G	0	1
4	15676	A→G	0	1
5	15681	G→A	0	1
6	15699	G→A	0	1
7	15725	A→G	0	1
8	15762	A→G	0	1
9	15781	T→C	0	1
10	15876	A→G	0	1
11	15446	T→C	0.84	0.16
12	15455	A→G	0.84	0.16
13	15473	A→G	0.72	0.28
14	16386	C→T	0.88	0.12
15	16387	A→G	0.92	0.08
16	16397	T→C	0.88	0.12

[a] *with reference to NC_012098*

Multidimensional scaling (MDS) plot of mitochondrial cytochrome-b, cytochrome-c and d-loop regions for *Antilope Cervicapra* was generated individually as shown in *Fig. 1*, *Fig. 2*, *Fig. 3* and collectively *(Fig. 4)*. The greater clustering of the *Antilope Cervicapra* samples indicated lower genetic variability. Overall significant genetic differences among species of deer were observed.

Pairwise evolutionary distance also showed that populations were significantly different from each other. Genetic variability and phylogenetic relationship within and between analyzed groups was evaluated, which was based on polymorphic loci from *Bovidae* and *cervidae*. The level of heterozygosity was relatively comparable in all evaluated groups of animals. The highest homozygosity was observed in all selected species of *Bovidae* and *cervidae* and differences were very low.

Phylogenetic based analysis of *Cytochrome-b, cytochrome-c* and *d-loop* of the gene sequences revealed that each species individuals comprise a distinct clade *(Fig. 5)*. This clade was evidently distinct from other species of deer. The neighbour-joining tree created from mitochondrial genes based data set from *cervidae* and *bovidae* showed clear differentiation.

DISCUSSION

In this study, significant reduction in genetic diversity of the *bovidae* and *cervidae* members were found from different regions of Pakistan. Both allelic richness and heterozygosity were lower in these populations compared to the other populations of this region, as previously reported [12-17]. This reduction in genetic diversity could be due to direct result of a combination of geographic isolation or due to small

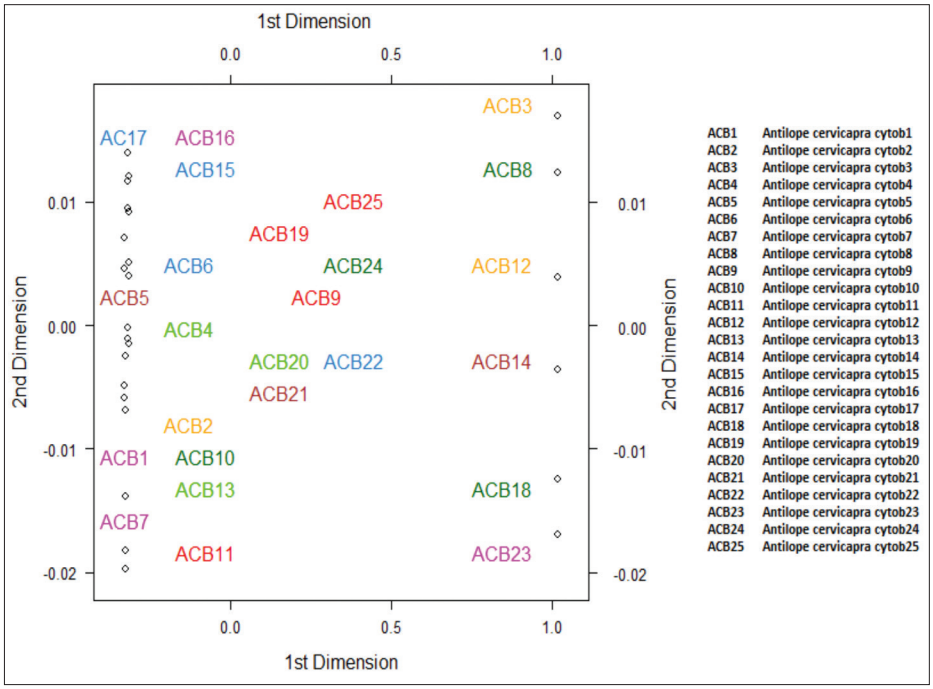

Fig 1. *cytochrome-b* based multidimensional scaling plot of *Antilope cervicapra*

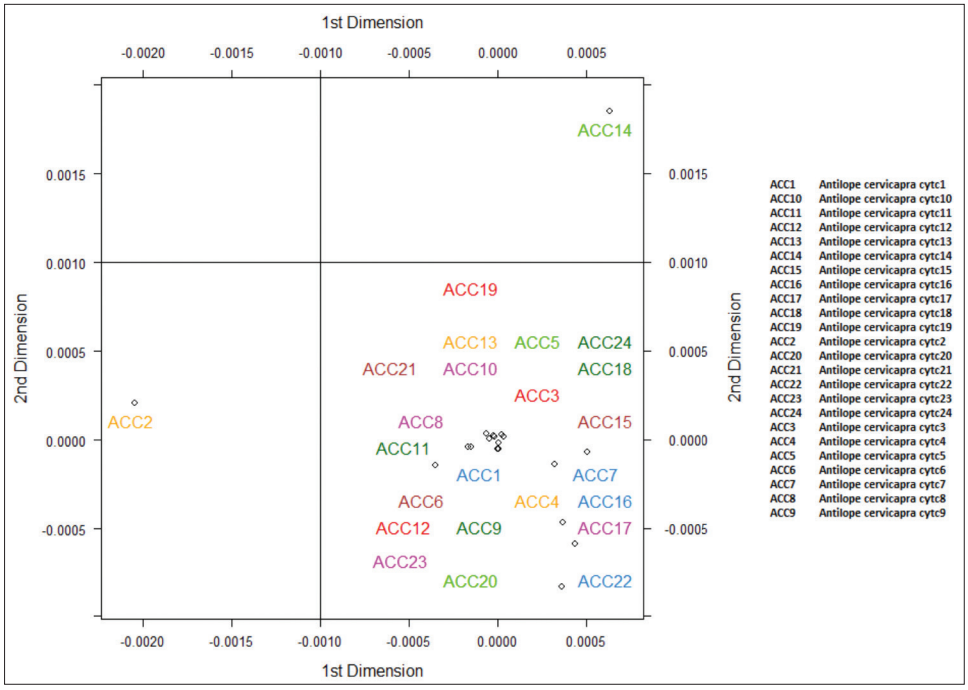

Fig 2. Multidimensional scaling plot of *cytochrome-c* gene for *Antilope cervicapra*

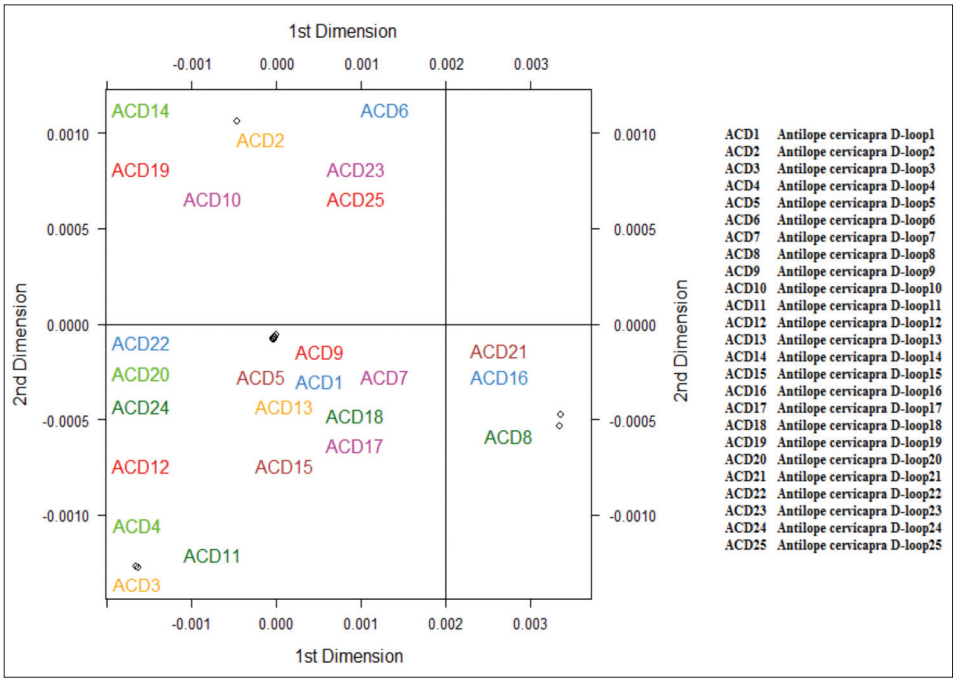

Fig 3. Multidimensional scaling plot of *d-loop region* for *Antilope cervicapra*

population sizes in the last decade. Similar observations have also been reported by Dellicour et al.[18] and Irwin et al.[19]. Phylogenetic analysis of Pakistani livestock breeds such as buffalo, goat, sheep, camel [4,20-24] have previously been reported but wildlife species data is scarce.

High mitochondrial DNA based genetic diversity was observed in Pakistani domestic goat's Indian blackbuck was successfully identified from other wildlife species of that region through DNA barcoding, which supports the findings of the current study. In another study on Japanese Sika deer *(Cervusnippon)*, demonstrated significant genetic diversity among other deer species through tandem repeats in D-loop of mitochondrial genome [23]. Phylogenetic analysis based upon a single gene is considered untrustworthy as far as topology is concerned, particularly in situations when larger classifications between species, such as interorder or interfamily, was under consideration. The reason was different evolutionary rates between different mitochondrial genes. So, phylogenetic analysis of *Cervidae* and *Bovidae* using more mitochondrial genes is needed. The phylogenetic analysis of this study supports other cladistics studies conducted on *antilopes* and *Bovidae* using DNA barcoding genes [24]. So, phylogenetic analysis

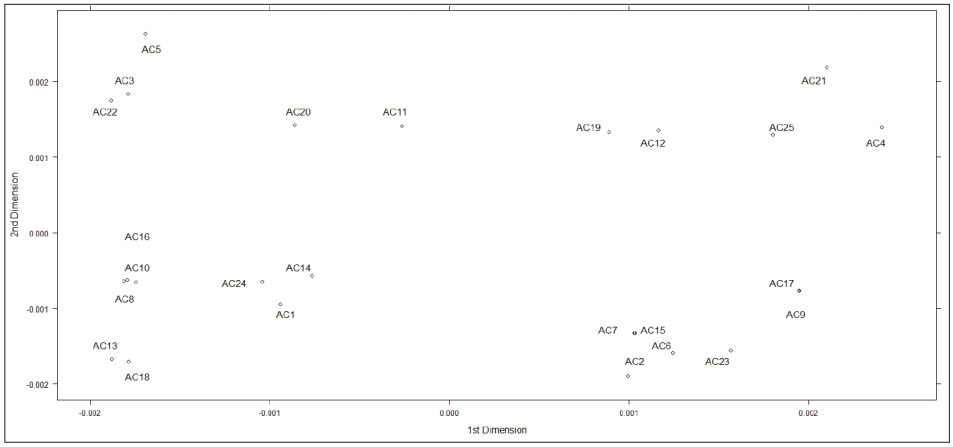

Fig 4. Multidimensional scaling plot of mitochondrial genomic *cytochrome-b, cytochrome-c* and *d-loop region (collectively)* for *Antilope cervicapra*

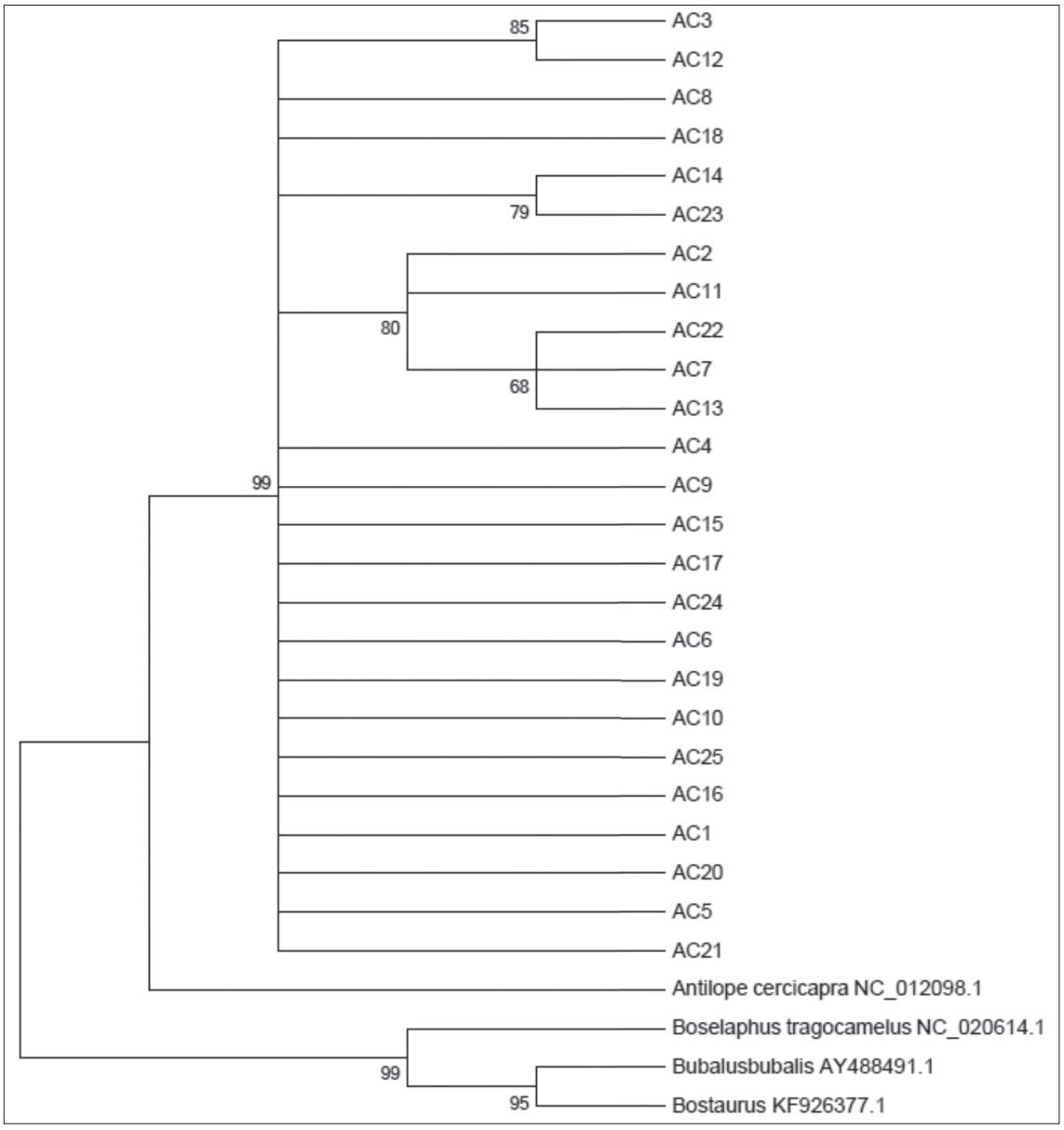

Fig. 5. Phylogenetic tree (rectangular) of *cytochrome-b, cytochrome-c and d-loop* region (collectively) of *Antilope cervicapra*

of *Cervidae* and *Bovidae* using more mitochondrial genes is needed. Another study regarding phylogeny analysis was conducted using *cytochrome b* gene, in different orders of eutherian animals including *Cervidae* and *Bovidae*, but they were not able to differentiate between *Cervidae* and *Bovidae* families. A phylogeny analysis was performed by Irwin et al.[19] and Honeycutt et al.[25] by using *cytochrome b* and *cytochrome c* oxidase subunit II (*COII*) genes in different species of mammals, including *Cervidae* and *Bovidae,* and different topologies were observed between *cytochrome b* and *COII* genes. According to Irwin et al.[19], on the basis of study of *cytochrome b* gene, *Cervidae* and *Bovidae* were not differentiated. The observations based upon the modern molecular genetics studies, recommend close relation of *Cervidae* with *Bovidae* then with *Moschidae*. But *Giraffidae* family appears more distant from *Cervidae* and closer to *Antilocapridae*.

Variations at genetic level is a bottom material for survival of animals. It act as the genetic source for prediction of future conservation of animals. Molecular markers plays initial guide for evaluation of the genetic variation. Therefore, the information on this level of genetic variation is prerequisite for designing effective strategy for wildlife conservation.

Overall, low level of generic diversity revealed with higher level of genetic differentiation among different populations. The genetic analysis showed the generic variation at species level. Combined effects of field and molecular techniques standardize the conditions for genomic amplification and analysis for status of population structure in *cervidae* species.

The mitochondrial genome sequence will directly facilitate conservation and genetic epidemiology research in *cervidae* family. Moreover, it will also assist in genome conservation of the endangered species of deer. The published mitochondrial genome sequenced data in NCBI database will serve as a source of valuable information to solve problem of specie identification and Blackbuck-related crimes in wildlife forensics.

Conflict of Interest

No conflict of interest has been found.

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