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EXPLORING THE GENETIC DIVERSITY AND PHYLOGENY OF FOUR NATIVE WILD DEER SPECIES OF PAKISTAN USING MITOCHONDRIAL MARKERS

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ABSTRACT

Bovidae and *Cervidae* are the most important families of mammals. Owing to extensive human interference, excessive use of natural resources, unregulated hunting, and other adverse environmental conditions, some of these wild species are now facing the risk of extinction. Genetic variation underpins animal survival from an evolutionary perspective, which is vital for the conservation of animals, particularly endangered species that, if not protected, will soon become extinct. Therefore, the current study was planned to investigate the molecular phylogeny and genetic diversity of *Bovidae (Boselaphus tragocamelus, Antilope cervicapra)* and *Cervidae (Axis axis, Axis porcinus)* families of wild deer in Pakistan using the collective effect of mitochondrial cytochrome b, cytochrome c oxidase subunit 1 (Cox1), and D-loop regions. For the genetic diversity study, we collected blood samples from unrelated wild deer of all four species (n=100; 25 per species) in EDTA-containing vacutainers. Genomic DNA was extracted using the phenol-chloroform method. PCR was performed to amplify the cytochrome b, Cox1, and D-loop genes, and the PCR products were sequenced in Foster City, CA, USA. In this study, we found evidence of a significant reduction in genetic diversity among members of *Bovidae* and *Cervidae* from different regions of Pakistan. The findings of the current study revealed the genetic distinctness of the studied wild deer species and their evolutionary divergence. Moreover, native deer populations exhibited reduced within-population genetic variability; therefore, a viable conservation plan is needed to ensure their survival and protection under changing environmental conditions.

Keywords: Genetic diversity, Wild deer, Cytochrome b, Cox1, and D loop.

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INTRODUCTION

Over time, ecosystem degradation from human activities has reduced biodiversity in plants and animals. Uncontrolled growth of the human population has led to increased consumption of natural products (Barbier, 2021); therefore, natural resources are depleting at a rate of 5% per decade (Seabury *et al*., 2004). This loss of the earth's biological wealth is catastrophic and has aesthetic, economic, and ecological implications. The loss of natural resources has widespread implications, especially for wild populations, as some are on the verge of extinction. The sixth mass extinction has begun, resulting in the extinction of many species, and thousands of species have become endangered, which poses a serious threat to Earth's environment (Ceballos *et al*., 2020).

Therefore, urgent attention is needed to conserve rare species through research and the collection of useful information about them. The loss of such species and their habitats has prompted conservationists and other environmental activists worldwide to protect this biodiversity by sharing knowledge and resources. On the contrary, this area has long been neglected in Pakistan. Hence, the current study is designed to fill this information gap and to understand the genetic diversity of four native wild deer species, e.g., *Boselaphus tragocamelus, Antilope cervicapra (Bovidae),* and *Axis axis* and *Axis porcinus (Cervidae).*

Recent molecular genetic studies on wild deer populations in Pakistan have begun to elucidate their genetic structure and conservation needs, providing a scientific basis for informed policy interventions. Analyses of mitochondrial *cytochrome b (cytb), cytochrome c* oxidase subunit 1 *(Cox1),* D-loop, and *ATPase* 6/8 genes in Hog deer (*Axis porcinus*) populations from Punjab have revealed low nucleotide diversity and significant phylogeographic structuring, suggesting population isolation and reduced gene flow (Arif *et al*., 2022). Similarly, sequence analysis of the mitochondrial D-loop region in Pakistani spotted deer (*Axis axis*) populations has indicated limited haplotype diversity, highlighting potential inbreeding and demographic bottlenecks (Abbas *et al*., 2016). The recent publication of a high-quality *A. porcinus* reference genome now enables genome-wide SNP discovery and demographic reconstructions, offering a robust platform for monitoring genetic health and planning translocations or captive-breeding programs (Wang *et al*., 2019). These findings underscore the urgency of incorporating molecular data into national and provincial conservation frameworks. Although Pakistan's National Biodiversity Strategy and Action Plan (NBSAP 2017–2030) and provincial wildlife laws formally recognize the conservation of species and genetic diversity as priorities, implementation remains fragmented and lacks systematic genetic surveillance (Government of Pakistan, 2017; Khan, 2021). Integrating routine molecular monitoring into provincial wildlife management, coupled with habitat connectivity restoration and anti-poaching measures, would significantly strengthen evidence-based conservation strategies for wild deer populations in the country.

Genetic diversity is vital for the survival of wildlife species. 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 (Thévenon *et al*., 2004). There is consensus among conservation geneticists that genetic diversity within and among populations is necessary for the survival (Hartl *et al*., 2003). Thus, assessment of genetic diversity is necessary for the maintenance of sustainable hunting, conservation and improvement of the genetic resources of animals of a specific population (Markov *et al*., 2015). For phylogenetic studies and species identification of different animals, *cytb* gene, along with other mitochondrial DNA markers, has been used frequently in the literature (Hsieh *et al*., 2001).

The literature review indicates that wild deer are a neglected population, and few molecular studies are available to elucidate the genetic diversity and population structure of native wild deer species in Pakistan. The available literature is limited, as most research has focused on one or two wild deer species, and genetic diversity has been studied only to a limited extent within these species. Moreover, understanding the various components of genetic diversity provides a baseline for the proper conservation of wild deer, which is now listed as endangered in Pakistan. Therefore, the current study is specifically designed to explore the genetic diversity of four native wild deer species in Pakistan using mitochondrial DNA markers. The aspects of genetic diversity studied will be useful for the effective conservation of native wild deer species in the country.

MATERIALS AND METHODS

Taxonomic Species and Sampling Strategy: Four species of wild deer, two from each *Bovidae* and *Cervidae* families, were selected for this study. A total of 100 animals (25 samples from each species) were selected for sampling. Blood samples were collected in EDTA-containing vacutainers from zoos, wildlife farms, and captive breeding centers across different regions of Pakistan. Sampling areas and geographical distribution of Blue, Black, Spotted, and Hog deer are provided in Supplementary Table 1. A pictorial representation of *Bovidae* and *Cervidae* members is provided in Figure 1.

DNA Extraction and Quantification: DNA was extracted using an organic method as described by Griffiths and Chacon-Cortes (2014). One milliliter of blood was collected, mixed with 2 mL of lysis buffer, thoroughly vortexed, and centrifuged at 1500 g (2690 RPM) for 5 minutes. This step was repeated with 3 mL of lysis buffer. The supernatant was discarded, and 300 µL of DNA extraction buffer was added, followed by 5 µL of Proteinase K (10 mg/mL) and 20 µL of 10% SDS. The samples were gently vortexed and incubated at 65 °C overnight. After incubation, samples were frozen at −20 °C until completely solidified, then thawed, and 120 µL of 5 M NaCl was added and mixed. Samples were centrifuged at 6000 RPM for 15 minutes. One milliliter of 100% ethanol was added to a new conical tube, and the clear supernatant containing DNA was transferred to obtain the pellet, which was finally resuspended in 50 µL of DNase/RNase-free water. DNA samples were resuspended at 4 °C overnight and then stored at −80 °C. The concentration and optical density of DNA samples

were measured using a NanoDrop spectrophotometer (Quawell Q5000).

Primer Designing: Three mitochondrial loci (*cytb*, *Cox1*, and D-loop regions) were amplified individually for all four species under study. Reference sequences of the complete mitochondrial genome, including *cytb*, *Cox1*, and D-loop regions for *Axis axis* (Accession No NC_020680), *Axis porcinus* (Accession No. JN632600), *Boselaphus tragocamelus* (Accession No.NC_020614), and *Antilope cervicapra* (Accession No. NC_012098) were taken from NCBI (www.ncbi.nlm.nih.gov). Primers were designed using NCBI Primer-BLAST.

PCR and DNA Sequencing: PCR was performed using a Bio-Rad thermocycler with locus-specific primers. The primers used for amplification are listed in Table 1. Each primer pair was applied to amplify DNA from the selected species (*Boselaphus tragocamelus*, *Antilope cervicapra*, *Axis axis*, and *Axis porcinus*). Amplification was performed at the annealing temperature specific to each primer, as shown in Table 1.

Table 1: List of primer sequences

Primer name	Locus	Primer sequence (5' to 3')	Product size (bp)	*TA* (°C)
CytoB-F	*cytb*	GTCATTCAACTACAAGAACACTA	1289	51
CytoB-R	*cytb*	TAAATAGAACTTCAGCTTTGGG		51
CytoC-F	*Cox1*	GCTTCAATCTACTTCTCCCG	1545	53
CytoC-R	*Cox1*	GTGGTTATGATGTTGGCTTG		53
DLF	D- loop	AGCCTCACTATCAACACCCA	1020	54
DLR	D- loop	CACATAGGTTTGGTCCCAGC		54

Figure 1: Pictorial representation of Nilgai or blue bull *Boselapus tragocamelus* Blackbuck *Antilope cervicapra (Bovidae),* Chital or spotted deer *Axis axis* and Hog deer *Axis porcinus (Cervidae).*

Amplification was carried out in a final volume of 25 µL containing forward and reverse primers, dNTPs (Deoxynucleoside Triphosphates), Taq buffer A, DNA Taq polymerase (Roche Diagnostics, Basel, Switzerland), RNase-free water, and template DNA. PCR products were sequenced using dye-labeled dideoxy terminator sequencing on an ABI Genetic Analyzer 3130 XL (Applied Biosystems Inc., Foster City, CA, USA) following standard protocols. For visualization, PCR products along with a 1 kb DNA ladder were electrophoresed on a 1.2% agarose gel at 100 V for 35 minutes. Amplification bands were visualized under UV light, compared with the DNA ladder, and documented using a gel documentation system.

Bioinformatics and Statistical Analysis: Sequences from all four species were aligned using BLAST 2 and Clustal W (IUB, International Union of Biochemistry). Phylogenetic analysis was conducted in MEGA2 using the maximum-likelihood method. Multidimensional scaling plots were generated using the Kimura 2-parameter (K80) model in R software, and genetic variation plots for all three genes of each species were also produced in R. Nucleotide diversity (π), haplotype diversity (h), number of polymorphic sites (S), Haplotype number (H) were estimated using DnaSP v6 from mitochondrial *cytb*, *Cox1*, and D-loop sequences.

RESULTS

The main objective of the study was to assess the collective impact of genetic variations in mitochondrial *cytb*, *Cox1*, and D-loop regions in *Boselapus tragocamelus* and *Antilope cervicapra (Bovidae)*, *Axis axis* and *Axis porcinus (Cervidae)* of Pakistan. Sequences of mitochondrial cytochrome *b*, *Cox1*, and mitochondrial D-loop regions for these four species were retrieved from NCBI (www.ncbi.nlm.nih.gov) to design primers. Mitochondrial regions of interest were amplified and sequenced bidirectionally using BigDyeTM Terminator on an ABI 3130XL Genetic Analyzer.

Phylogenetic analysis: The current study investigated the genetic diversity of wild deer by generating phylogenetic trees and multidimensional scaling plots based on the *cytb*, *Cox1*, and D-loop regions of mitochondrial DNA. Analyses were first conducted separately for two *Bovidae* species and two *Cervidae* species, followed by a combined analysis of all four species.

DNA sequences of *cytb*, *Cox1*, and D-loop genes clearly distinguished the studied wild deer populations into four distinct clades. Figures 2, 4, and 6, representing the respective phylogenetic trees, illustrate the evolutionary divergence of *Boselaphus tragocamelus*, *Antilope cervicapra*, *Axis axis*, and *Axis porcinus* based on *cytb*, *Cox1*, and D-loop sequences, respectively. All analyses consistently showed clear separation of the four species into distinct clades with tightly clustered branches. The branching patterns indicate that each species maintained a strong genetic identity, with minimal overlap observed between groups.

In addition to the clear clade separation, interspecific branch lengths corresponded to substantial mitochondrial divergence, with average Kimura 2-parameter genetic distances ranging from ~0.15 to 0.22 among species, whereas within-species distances remained consistently low (<0.02), indicating high intra-specific genetic homogeneity.

Branch lengths in the phylogenetic trees reflected genetic distances, with shorter branches indicating closer genetic similarity within species and longer, inter-clade branches representing deeper evolutionary divergence. The trees exhibited distinct clustering of each species into separate clades, demonstrating clear genetic differentiation and independent evolutionary lineages. Similarly, Figures 3, 5, and 7, which depict circular phylogenetic trees, provided a more detailed view of the same dataset based on cytb, Cox1, and D-loop sequences, highlighting both intra- and interspecies relationships. Overall, sequences of the studied genes formed well-defined clades for each species, revealing their genetic distinctness and evolutionary divergence among the wild deer species.

MDS Analysis of Variation:

Mitochondrial *cytb, Cox1, and* D-loop region-based analysis of *Boselaphus tragocamelus and Antilope cervicapra (Bovidae):* To further explore the genetic diversity of the studied wild deer *cytb, Cox1,* and D-loop based multidimensional scaling plot was generated for two species of *Bovidae (Boselaphus tragocamelus* and *Antilope cervicapra)* (figure 8), and pairwise distances were calculated for these two species (figure 9). The plot was constructed using 1st and 2nd-dimensional transformations, showing symmetric variation in genetic distance values in the MDS plot. Quantitatively, *Antilope cervicapra* individuals exhibited near-zero pairwise genetic distances (<0.01), whereas *Boselaphus tragocamelus* showed markedly higher mean genetic distances (~0.20), reflecting strong mitochondrial divergence between the two *Bovidae* taxa.

The MDS plots revealed distinctness of the studied populations (species) and clear clustering, demonstrating high within-species genetic homogeneity and strong between-species genetic differentiation. The combined *cytb, Cox1,* and D-loop sequence-based genetic variation plot of the studied species revealed an evident interspecific divergence in which *Antilope cervicapra* individuals showed near-zero genetic distances, whereas *Boselaphus tragocamelus* individuals showed consistently higher genetic distances (~0.20), indicating

marked genetic separation between the two taxa. The clear discontinuity in genetic distance suggested limited or no gene flow and confirms their separate evolutionary lineages within *Bovidae*.

Mitochondrial *cytb, Cox1,* and D-loop region-based (collective) analysis of *Axis axis,* and *Axis porcinus (Cervidae):* *A cytb, Cox1,* and D-loop-based multidimensional scaling plot was generated for two species of *Axis axis* and *Axis porcinus (Cervidae),* as demonstrated in Figure 10, and pairwise distances were calculated for these two species (Figure 11). The MDS plots demonstrated clear separation clustering of the studied species, and genetic variation plots showed marked genetic separation between the two taxa. *Axis axis* and *Axis porcinus* formed distinct clusters with moderate interspecific genetic distances (approximately 0.10–0.15), while within-species distances remained low (<0.03), supporting clear species-level separation with limited intra-population variability.

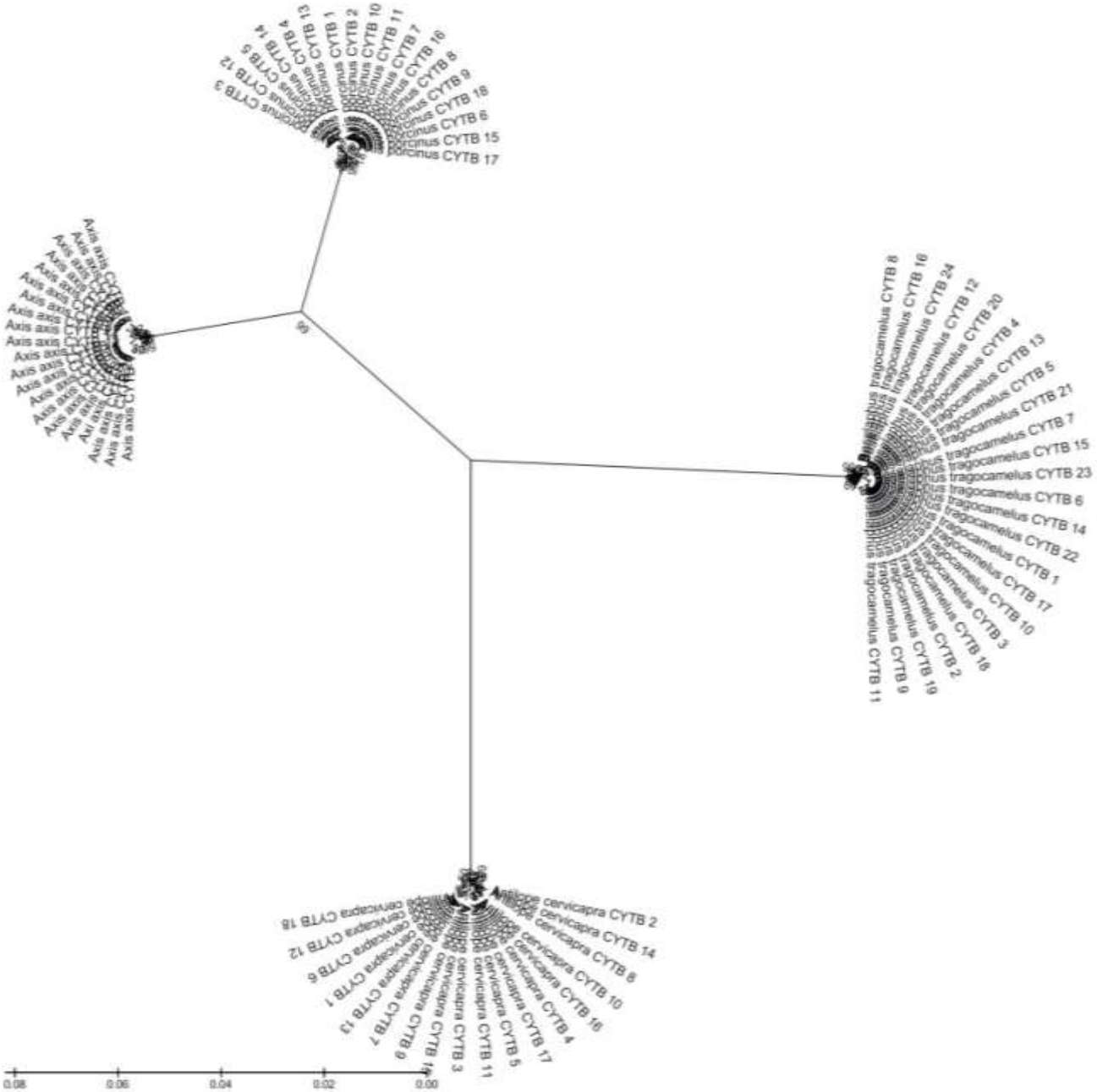

Figure 2: Phylogenetic tree (radiation) of the *cytb* gene of studied four wild deer species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus).*

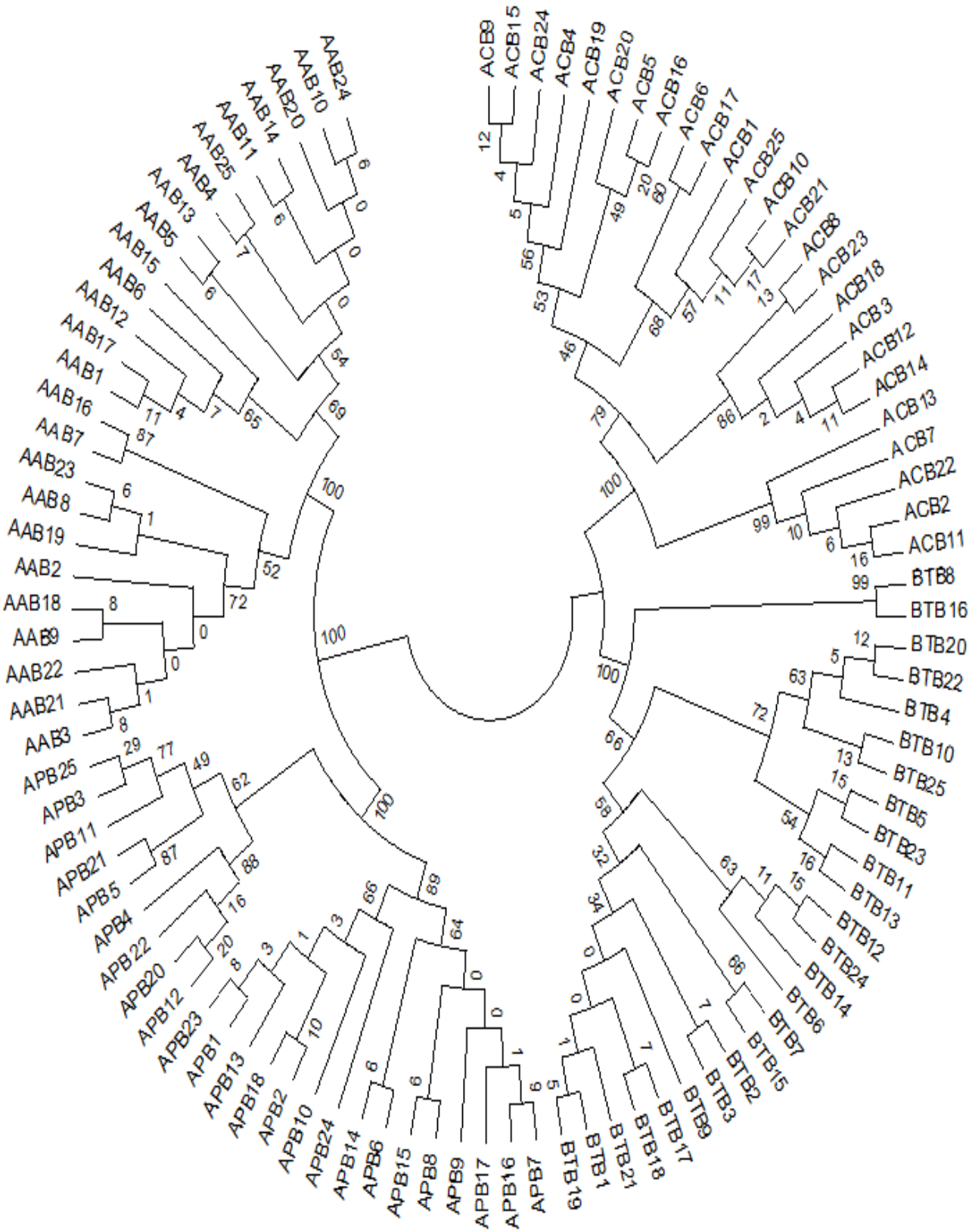

Figure 3: Phylogenetic tree (circular) of the *cytb* gene (collectively) of the studied four wild deer species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis, and Axis porcinus)*.

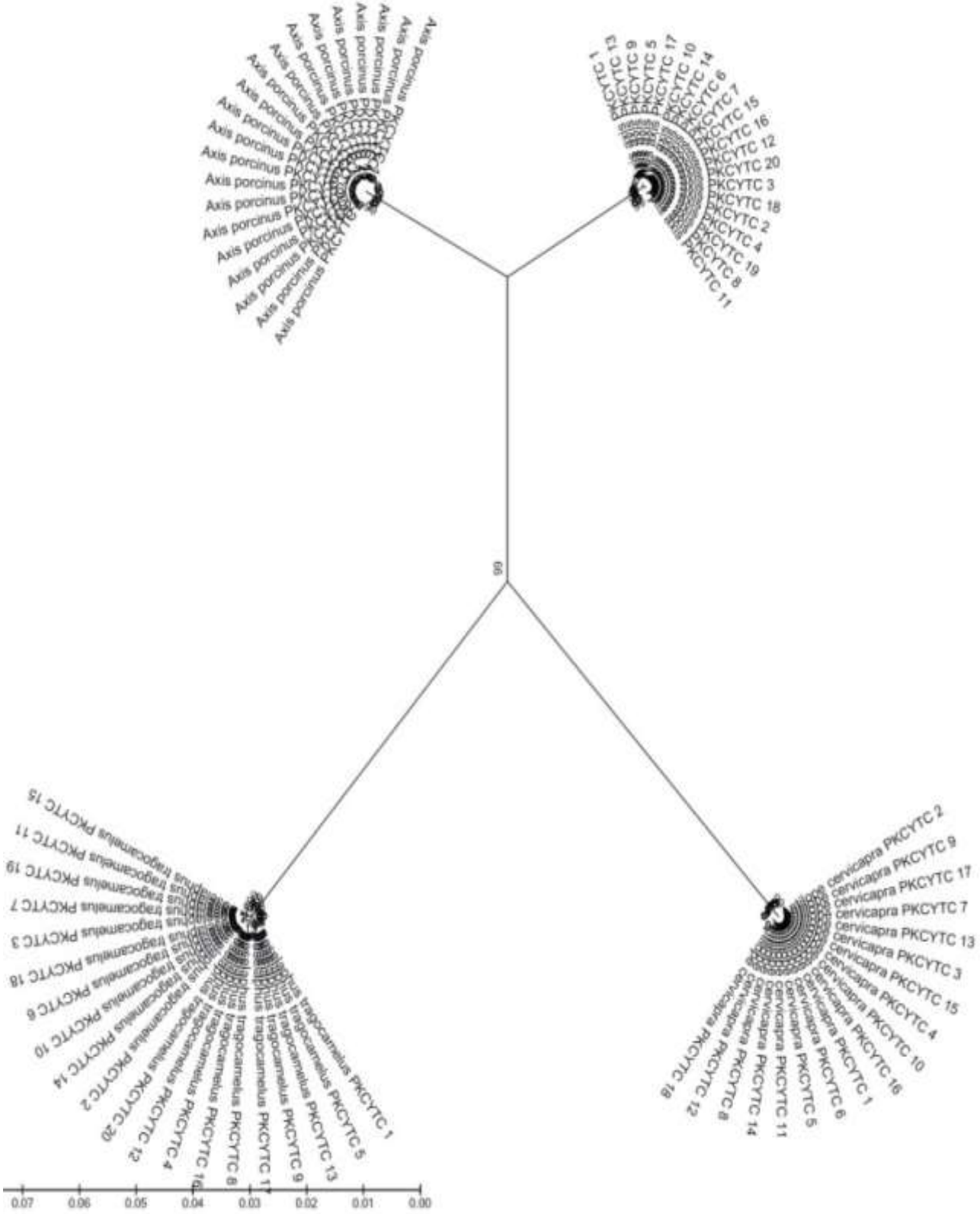

Figure 4: Phylogenetic tree (radiation) of the *Cox1* gene (collectively) of the studied species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus).*

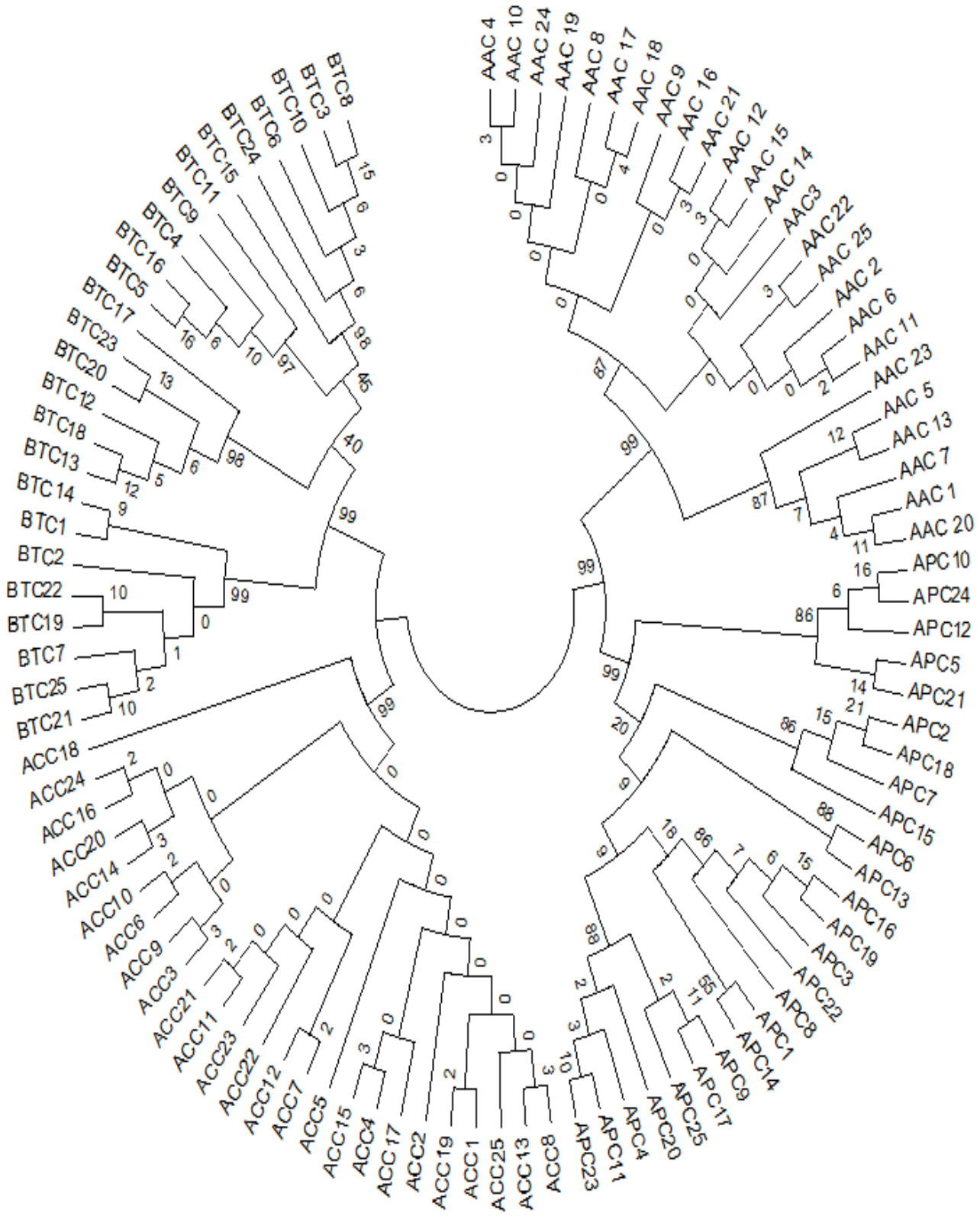

Figure 5: Phylogenetic tree (circular) of the *Cox1* gene of the studied species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis, and Axis porcinus).*

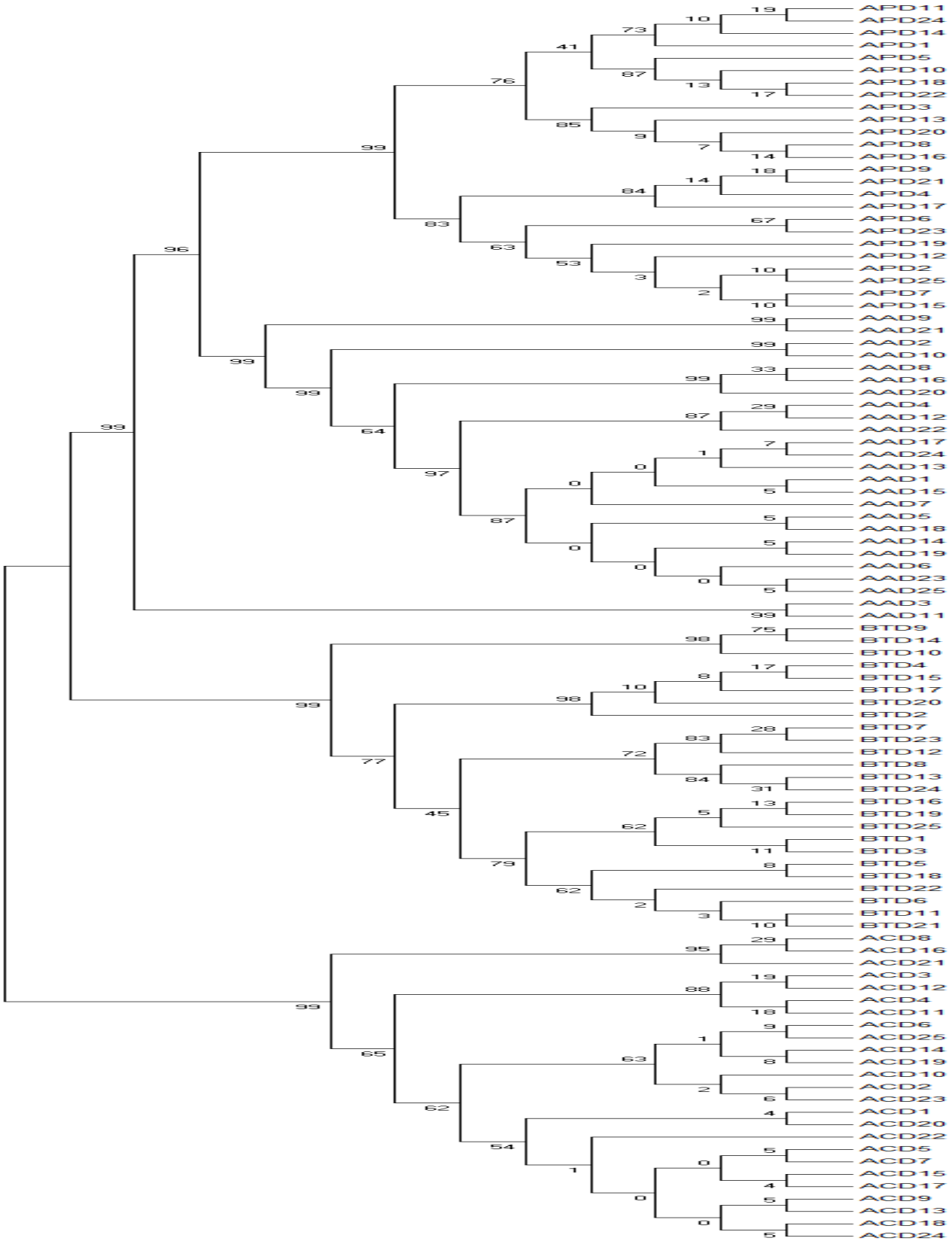

Figure 6: Phylogenetic tree (rectangular) of the D-loop region of the studied species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus).*

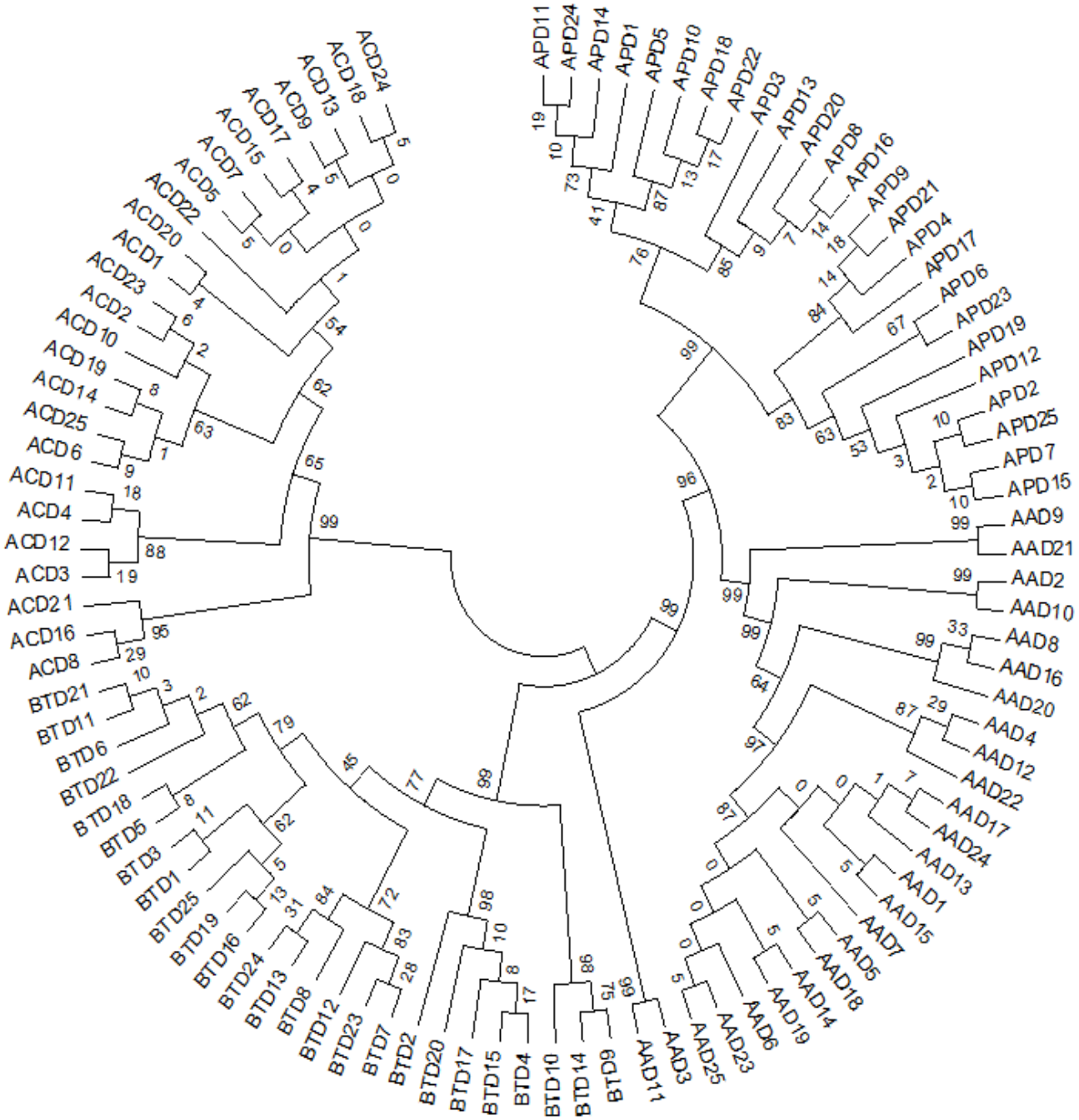

Figure 7: Phylogenetic tree (circular) of the D-loop region of the studied species (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis, and Axis porcinus).*

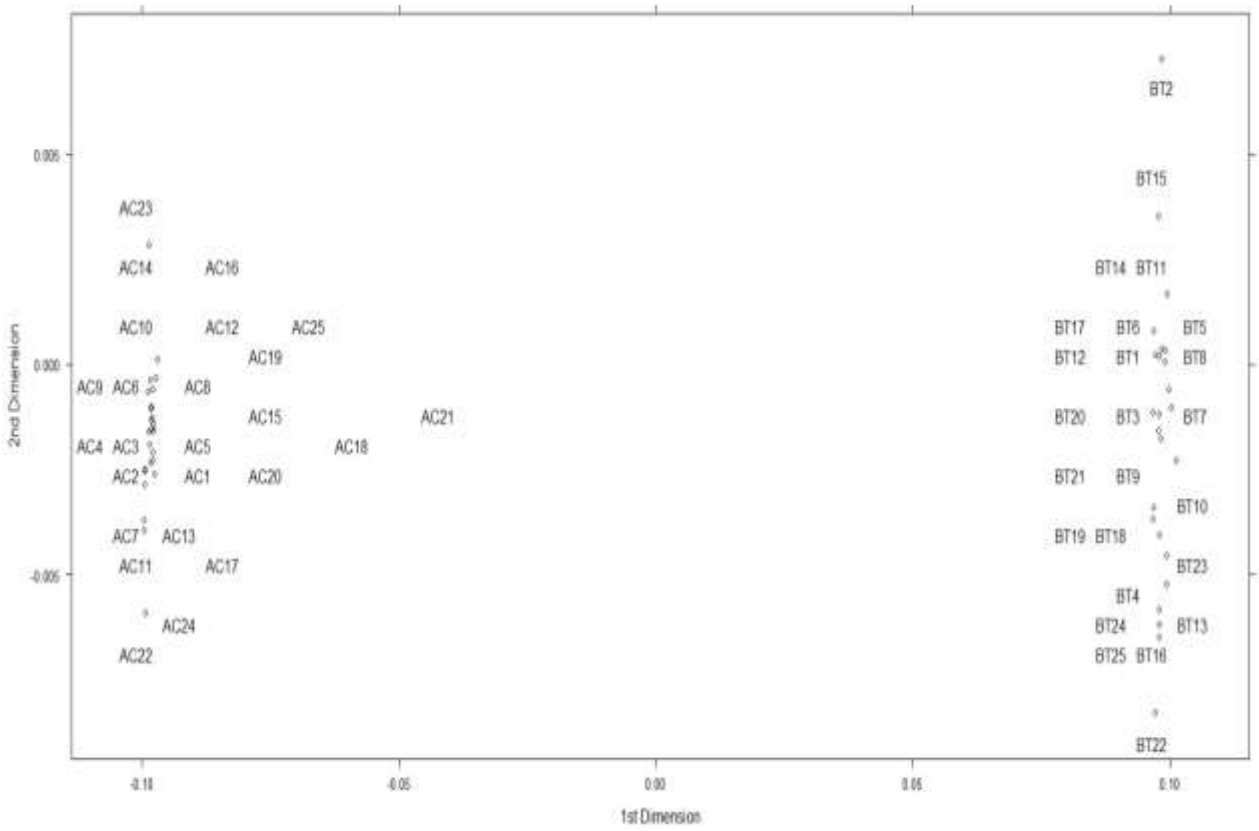

Figure 8: Multidimensional scaling plot of *cytb, Cox1,* and D-loop *region* for *Bovidae (Boselaphus tragocamelus* and *Antilope cervicapra)*.

Pairwise Evolutionary Distance

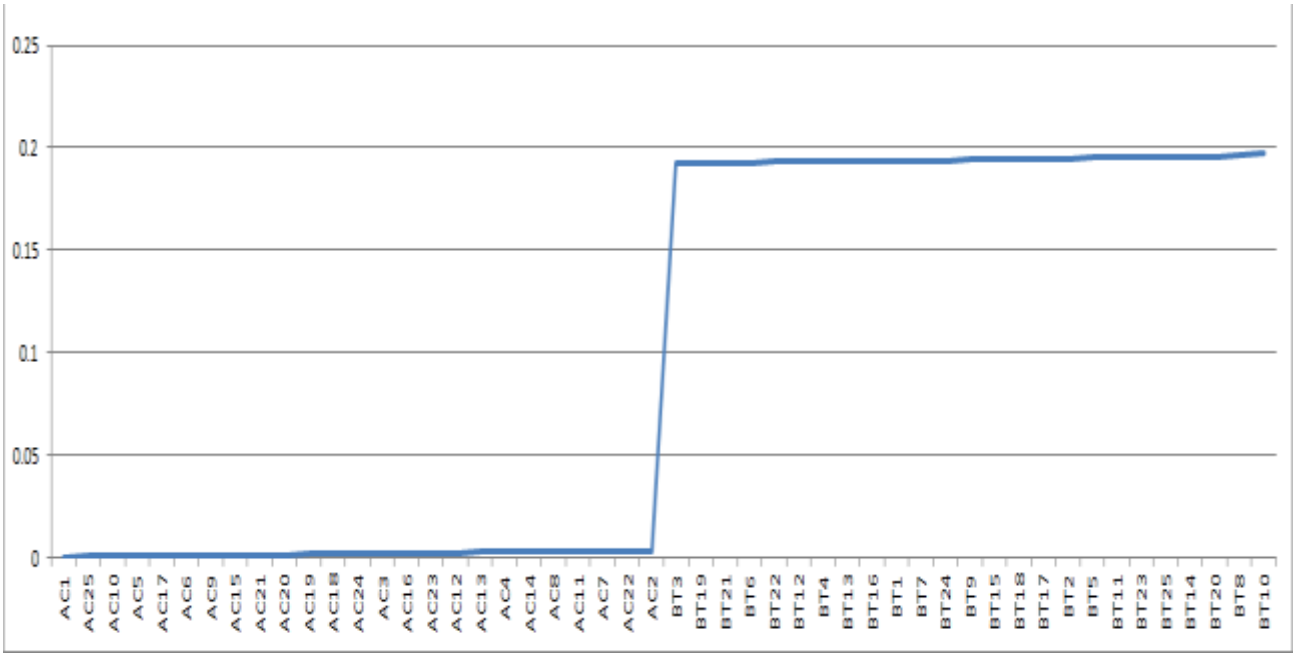

Figure 9: *Cytb, Cox1,* and D-loop region-based genetic variation plot of *Bovidae (Boselaphus tragocamelus* and *Antilope cervicapra)*

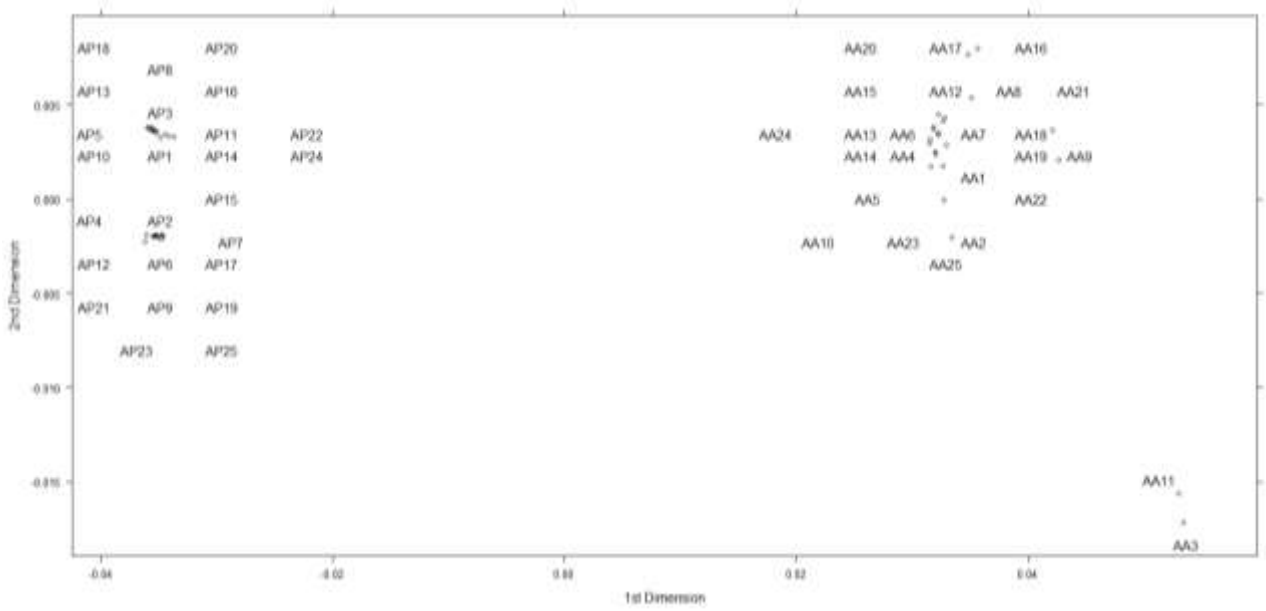

Figure 10: Multidimensional scaling plot of *cytb, Cox1,* and D-loop for *Cervidae (Axis axis* and *Axis porcinus)*

Pairwise Evolutionary Distance

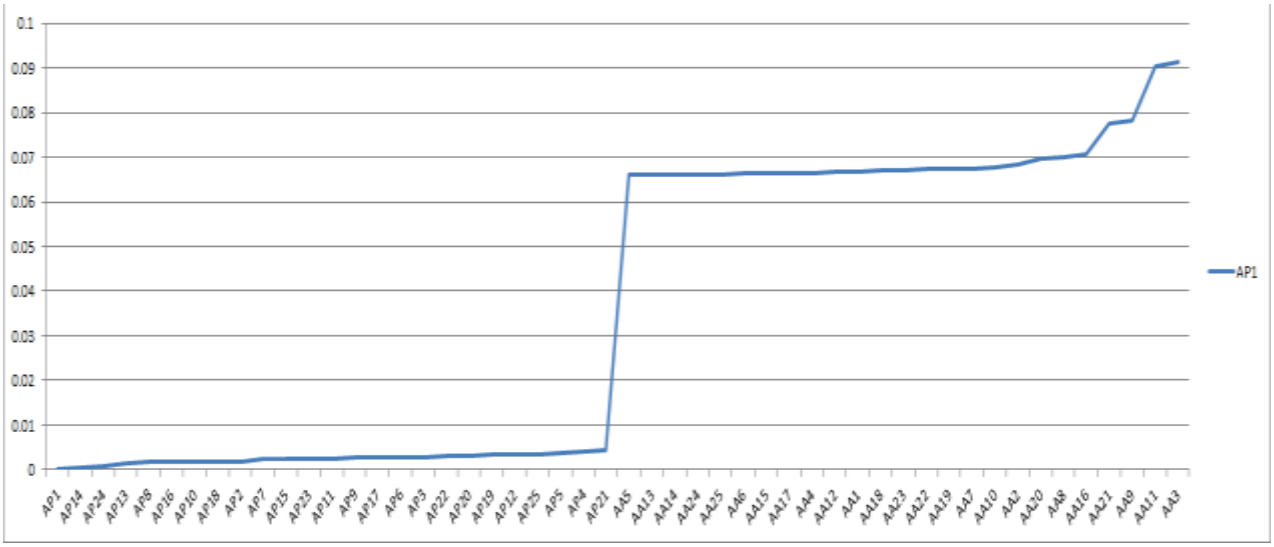

Figure 11: *Cytb, Cox1,* and D-loop region-based genetic variation plot of *Cervidae (Axis axis* and *Axis porcinus)*

Mitochondrial *cytb, Cox1,* and D-loop region-based analysis of *Boselaphus tragocamelus, Antilope cervicapra (Bovidae), Axis axis,* and *Axis porcinus (Cervidae):* A *cytb, Cox1*, and D-loop-based multidimensional scaling plot was generated for all 4 species of wild deer under study, as demonstrated in Figure 12, and pairwise distances were calculated for the studied species (Figure 13). The clear intra-specific clustering and wide inter-cluster distances revealed high genetic homogeneity within each species and strong mitochondrial genetic partitioning among the four taxa, thereby supporting their clear evolutionary separation. However, *Antilope cervicapra* (AC) and *Axis axis* (AAB) form partially overlapping individuals, suggesting moderate genetic divergence, yet some phylogenetic closeness was observed within *Cervidae*. Across all four taxa, interspecific genetic distances ranged from ~0.12 to 0.25, with the greatest divergence observed between *Boselaphus tragocamelus* and *cervid* species, whereas partial overlap noted between *Antilope cervicapra* and *Axis axis* corresponded to comparatively lower but still distinct genetic distances (~0.10).

Rectangular phylogenetic tree: Moreover, a rectangular phylogenetic tree using combined sequences of *cytb,*

Cox1, and the D-loop region of *Boselaphus tragocamelus*, *Antilope cervicapra*, *Axis axis*, and *Axis porcinus* was generated as presented in Figure 14. The tree clearly depicted the four studied species, i.e., *Boselaphus tragocamelus* (BT), *Antilope cervicapra* (AC), *Axis axis* (AAB), and *Axis porcinus* (AP) as distinct monophyletic clades, demonstrating their evolutionary segregation. The BT forms a deeply diverged branch that clusters separately from all cervid taxa, reflecting its distinct phylogenetic position within *Bovidae*. Within *Cervidae*, AC and AAB form closely related but discrete sister clades, indicating their shared evolutionary ancestry yet sufficient genetic divergence to maintain species-level separation. AP emerges as a distinct clade branching earlier than AC and AAB, signifying substantial mitochondrial divergence from the other cervids. The combined mitochondrial dataset further confirmed these patterns, with deep divergence of *Boselaphus tragocamelus* (genetic distance >0.20) relative to *cervids*, while Axis porcinus diverged earlier within *Cervidae*, showing greater mitochondrial separation than *Axis axis* and *Antilope cervicapra*. Conclusively, the phylogenetic tree confirmed strong interspecific genetic differentiation and evolutionary distinctness among the four taxa.

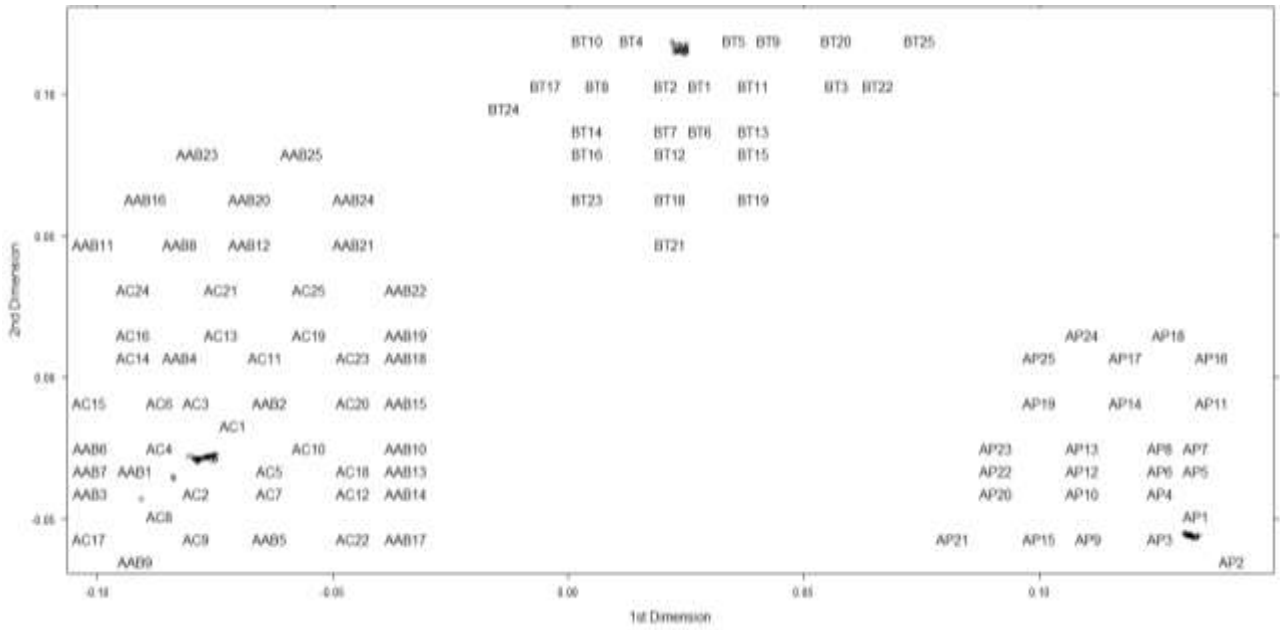

Figure 12: Multidimensional scaling plot of *cytb, Cox1,* and D-loop region for all four native wild deer species.

Pairwise Evolutionary Distance

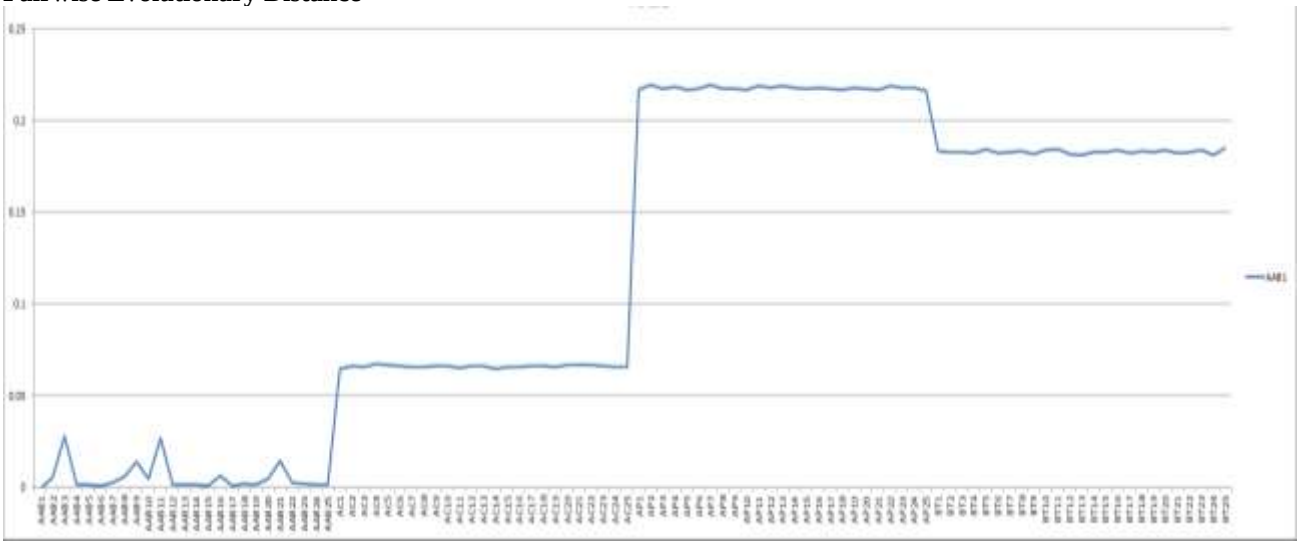

Figure 13: *Cyb, Cox1,* and D-loop region-based variation plot of the studied four wild deer species.

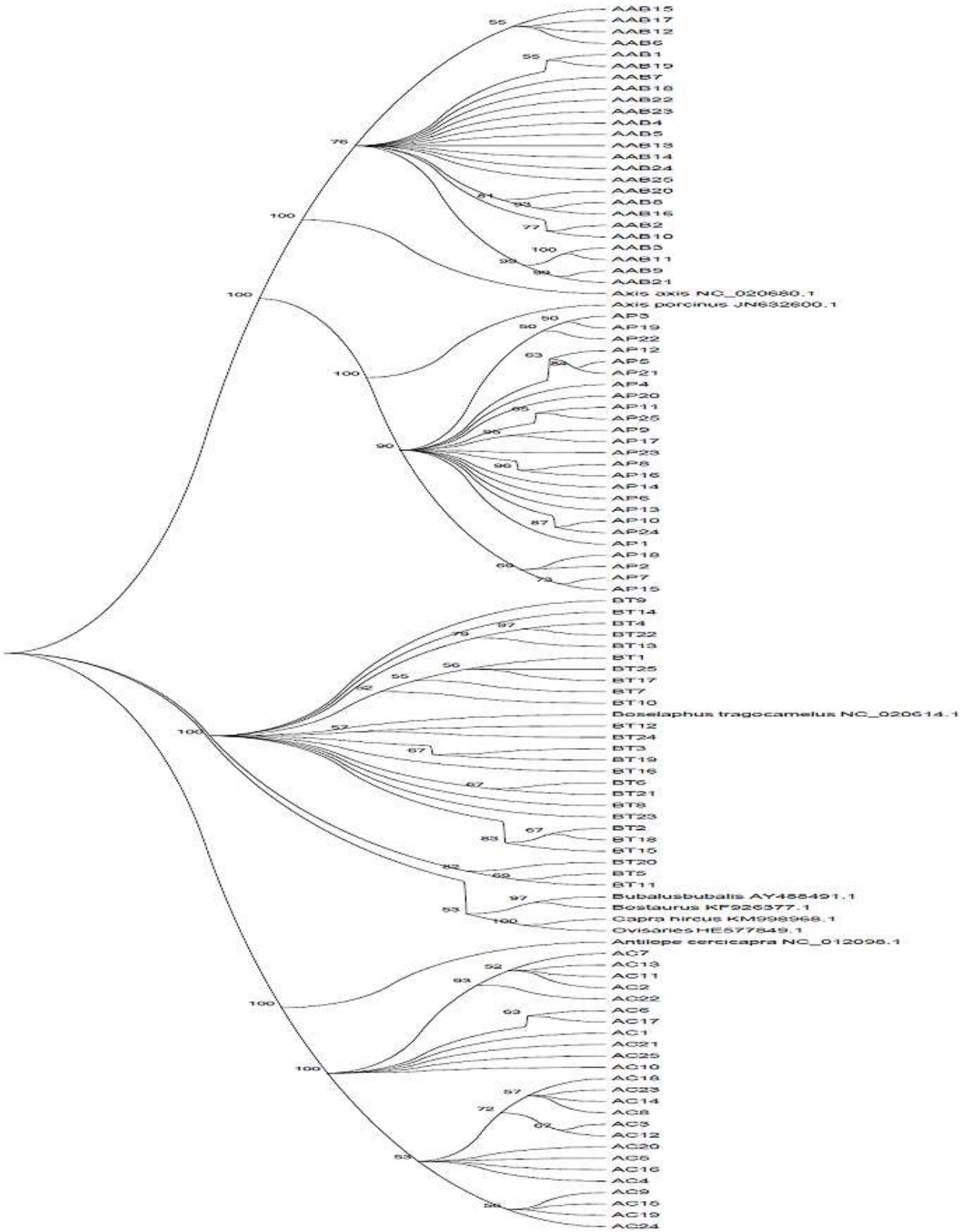

Figure 14: Phylogenetic tree (Rectangular) of *cytb, Cox1,* and D-loop region of *Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus.*

Mitochondrial genetic diversity estimates: The present study provides the first comprehensive mitochondrial genetic assessment of four native wild deer species of Pakistan (*Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus*) using combined cytb, *Cox1*, and D-loop markers. Despite clear clustering and population differentiation among the studied wild deer species, quantitative analyses revealed consistently low mitochondrial genetic diversity, characterized by a limited number of haplotypes and low nucleotide diversity ($\pi \approx 0.001–0.002$). Notably, *Antilope cervicapra* and *Boselaphus tragocamelus* exhibited particularly low haplotype and nucleotide diversity, indicating critically reduced within-population genetic variability (Table 2).

Table 2. Mitochondrial genetic diversity indices of four native wild deer species of Pakistan based on combined *cytb, Cox1,* and D-loop sequences

Species	Family	N	Combined sequence length (bp)	Haplotypes (H)	Polymorphic sites (S)	Haplotype diversity (h ± SD)	Nucleotide diversity ($\pi \pm$ SD)
Boselaphus tragocamelus	Bovidae	25	3,950	3	14	0.214 ± 0.061	0.0019 ± 0.0004
Antilope cervicapra	Bovidae	25	3,950	2	9	0.146 ± 0.052	0.0012 ± 0.0003
Axis axis	Cervidae	25	3,950	4	18	0.298 ± 0.074	0.0026 ± 0.0005
Axis porcinus	Cervidae	25	3,950	3	15	0.236 ± 0.068	0.0021 ± 0.0004

DISCUSSION

It is the first study of its kind on the molecular phylogeny and diversity analysis of *Bovidae (Boselaphus tragocamelus, Antilope cervicapra)* and *Cervidae (Axis axis, Axis porcinus)* in Pakistan using the combined effect of *cytb, Cox1,* and D-loop region mitochondrial markers. Mitochondrial markers such as *cytb, Cox1,* and D-loop region were widely used in deriving information on phylogenetic relationships and genetic diversity studies (Giovambattista *et al*., 2001; Féral *et al*., 2006; Mwacharo *et al*., 2006). Genome conservation plays an important role in the survival of species and ecosystems. The conservation of wild endangered species depends on the maintenance of genetic variations, which is necessary for the survival of these species (Lynch and Milligan, 1994; Izza *et al*., 2022).

The mechanisms of evolutionary history and the diversity of genes and populations can be better understood through reliable phylogenetic analysis. Another important feature is the development of a sound conservation and breeding strategy based on phylogeny. Suitable breeding objectives can be formulated based on genetic diversity parameters (Tabbaa and Al-Atiyat, 2009). Genetic variability and phylogenetic relationships within and between the analysed groups were evaluated using polymorphic loci from *Bovidae* and *Cervidae*. The level of heterozygosity was comparable across all evaluated animal groups. The highest homozygosity was observed across all selected Bovidae and Cervidae species, with very small differences among them. Pairwise evolutionary distance also indicated that populations differed significantly from one another.

Cytochrome-b, Cox1, and D-loop-based phylogenetic analysis of the gene sequences revealed that each species individually comprises a clade that is clearly distinct from the clade comprised of other deer selected for this study. The neighbor-joining tree constructed from mitochondrial genes, based on data from Cervidae and Bovidae, showed clear differentiation among *Boselaphus tragocamelus, Antilope cervicapra, Axis axis,* and *Axis porcinus*. The findings of this study indicated that these four deer species exhibit significant genetic variation that distinguishes them from one another.

We found evidence of a significant reduction in genetic diversity among members of the families *Bovidae* and *Cervidae* across different regions of Pakistan. Genetic differentiation within the individuals of the wild deer species was found to be lower compared to the larger populations from other regions previously reported by Frantz *et al*. (2006, 2008), Hmwe *et al*. (2006), Zachos *et al*. (2007) and Pérez-Espona *et al*. (2008). This reduction in genetic diversity is likely the direct result of geographic isolation and small population sizes that occurred in the recent past. Similar observations were also reported by Dellicour *et al*. (2011).

Based on the characterization of individual species using their mitochondrial markers separately, it was observed that high within-breed homogeneity and quite lower genetic differentiation were observed, which is comparable with the findings of (Abbas *et al*., 2016). Phylogenetic analysis of Nilgai revealed conserved neighboring patterns among different individuals, as they shared common ancestry (Abbas *et al*., 2017b). Lower genetic diversity was observed among the samples of the Hog deer (Abbas *et al*., 2017b). However, significant genetic variation was observed among other species and *Antilope cervicapra*. Phylogenetic analysis revealed a

distinct clade of this species compared to other species of deer (Abbas *et al*., 2017a).

Multidimensional scaling (MDS) plots of mitochondrial *cytb, Cox1,* and D-loop region for *Boselaphus tragocamelus, Antilope Cervicapra, Axis axis,* and *Axis porcinus* were generated. Single gene and three genes combined sequences based MDS plots were made in such a way: i) each species of deer, ii) *Boselaphus tragocamelus* and *Antilope cervicapra (Bovidae)*, iii) *Axis axis* and *Axis porcinus (Cervidae)*, and iv) *Boselaphus tragocamelus* and *Antilope cervicapra* (*Bovidae*) and *Axis axis* and *Axis porcinus* (*Cervidae*).

A cytochrome b, Cox1, and D-loop-based MDS plot for *Boselaphus tragocamelus* indicated that the samples clustered. The clustered samples pattern showed less genetic variability. The greater clustering of the samples indicates lower within-population genetic variability. The same pattern was observed when a combined sequence of three gene-based plots was constructed.

An overlap of individuals was observed in the overall genetic profile of the *Axis axis*, as identified by analysis. The greater clustering of the *Axis porcinus* samples also indicates lower genetic diversity. Comparison of the combined sequence-based genetic profiles of *Boselaphus tragocamelus* and *Antilope cervicapra* revealed that the amount of genetic variation in each population is similar to that in other populations, but both species of *Bovidae* and *Cervidae* differ significantly. Similarly, genetic variation was found to be significant between two species of the *Cervidae* family. Overall, significant genetic differences were observed between species, revealing their distinctiveness. However, a viable breeding strategy is needed to properly conserve these wild deer species.

Genetic diversity explores important information regarding the population structure of species and provides a basis for animal survival to launch a viable conservation program for the future (Muner *et al*., 2021). Molecular markers play the initial guide for evaluation of the genetic variation (Allendorf *et al*., 2010). Therefore, the information regarding genetic variation is the most important prerequisite for designing effective strategies for future conservation programs, especially in wild species that are on the verge of extinction currently (Crandall *et al*., 2000).

Recent studies from neighboring South Asian countries corroborate our findings, documenting reduced genetic diversity and pronounced interspecific differentiation in *Axis axis*, *Axis porcinus*, *Boselaphus tragocamelus,* and *Antilope cervicapra*. In central and southern India, Sathyakumar *et al*. (2020) reported high genetic structuring and low genetic diversity among fragmented populations of *Axis axis*, ascribing this pattern to evolutionary habitat fragmentation and limited dispersal, findings that align with our current study. Correspondingly, in western India, Sharma *et al*. (2022) observed marked mitochondrial lineage divergence and reduced nucleotide diversity in *Antilope cervicapra* populations, evidencing genetic bottlenecks in isolated populations.

Moreover, mitochondrial control region sequences from wild *Axis porcinus* populations in Nepal revealed low genetic variation and marked population differentiation. Kandel *et al*. (2021) analyzed and found low genetic variation and significant population differentiation among Terai populations, consistent with small effective population sizes.

In Iran, Ghaffari *et al*. (2023) documented strong phylogeographic structuring and low haplotype diversity in *Boselaphus tragocamelus* populations, suggesting that habitat loss and hunting pressure have led to severe demographic contractions. Collectively, these studies support the observation from our dataset that regional deer populations exhibit reduced within-population genetic variability and high between-population divergence, likely driven by prolonged isolation and anthropogenic pressures.

The comparison of our results with recent studies underscores the need for coordinated transboundary conservation efforts and genetic preservation plans to mitigate further genetic erosion in ecologically important ungulates across South Asia. However, the current study has several limitations, including a small sample size, reliance on mitochondrial markers only, and insufficient partitioning between captive- and wild-origin samples, which may obscure natural structure. The measured genetic diversity values are not fully representative of wild populations. To effectively assess genetic diversity and conserve native wild deer species in Pakistan, we recommend using a broad marker panel and a large reference population.

Conclusion: The mitochondrial genome sequence revealed important information on conservation, population genetics, management, and genetic research in *Bovidae* and *Cervidae*. Results indicated reduced mitochondrial genetic diversity across all species characterized by a limited number of haplotypes and low nucleotide diversity values ($\pi \approx 0.001–0.002$). Notably, *Antilope cervicapra* and *Boselaphus tragocamelus* exhibited particularly low haplotype and nucleotide diversity, indicating critically reduced within-population genetic variability, which raises concerns about the long-term survival of these species. The observed genetic erosion likely reflects prolonged population isolation, small effective population sizes, habitat fragmentation, and anthropogenic pressures, including unregulated hunting and restricted gene flow. Despite clear genetic differentiation among species, the low within-species diversity raises serious concerns regarding adaptive

potential, disease resistance, and long-term population viability under changing environmental conditions. These findings have direct conservation implications. We strongly recommend the immediate incorporation of genetic monitoring into national and provincial wildlife management programs, the prioritization of habitat connectivity restoration among fragmented populations, and the use of scientifically guided translocation or captive breeding strategies to prevent further genetic erosion. Without targeted conservation interventions, continued loss of genetic diversity may compromise the evolutionary resilience and survival of Pakistan's native wild deer populations. Therefore, further research using larger sample sizes and broader geographic coverage is required to develop effective conservation strategies for these species before their decline becomes irreversible.

List of Abbreviations

DNA: Deoxyribonucleic Acid
dNTPs: Deoxy nucleoside tri-phosphates
Kb: Kilobase
PCR: Polymerase chain reaction
BLAST: Basic local alignment search tool
MDS: Multidimensional scaling
NCBI: National Center for Biotechnology Information
UV: Ultraviolet

Conflict of interest: The authors declare no conflict of interest.

Ethics Statement: The study was approved by the Advanced Studies and Research Board of the University of Veterinary and Animal Sciences, Lahore, Pakistan. Blood samples were collected with the owners' and curators' consent. No animals were harmed during sampling and data collection.

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Author's Contribution: AN and MEB conceptualized the study, helped in data analysis, and supervised the research. GA wrote the initial draft. RDM, AHS, BM, MS, SAK, and YB wrote and revised the manuscript. All authors approved the final version.

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