RESEARCH ARTICLE

Genetic Diversity of Small, Isolated and Fragmented Blackbuck (*Antilope cervicapra,* L.) Populations of Haryana based on mtDNA Barcoding

Vikram Delu[1], Dharmbir Singh[1], Kanisht Batra[2]*, Sushila Maan[2], Deepesh Saini[3]

Abstract

Mitochondrial DNA (mtDNA) analysis is a valuable tool in forensic for species identification because mtDNA is maternally inherited, and there are often many copies of mtDNA in a cell. This makes it possible to obtain DNA from degraded or small samples, such as those found in forensic investigations. Additionally, mtDNA analysis can provide information on the evolutionary relationships among different populations and species. In the present study, mtDNA analysis was employed to examine the genetic diversity and phylogenetics of small Blackbuck populations in Haryana, India. The six tissue and eleven fecal samples were collected and amplified with the cytochrome c oxidase subunit I gene. Various statistical approaches were used to examine the parameters like Genetic p-distance, Genetic diversity, Tajima's D, Fu's Fs Test, Mismatch distribution graphs. The overall value of $\pi = 0.013$ and Hd = 0.65 of all selected populations suggested that the population of Blackbuck under investigation may have an equilibrium stage. The findings of present investigation provide insight into the genetic diversity and highlight the importance of maintaining connectivity among fragmented populations. This information is crucial for designing effective conservation strategies, as well as for addressing issues associated with species identification and combating wildlife-related crimes.

Key words: Blackbuck, Cytochrome c, Mitochondrial DNA, Wildlife Forensic.

Ind J Vet Sci and Biotech (2024): 10.48165/ijvsbt.20.5.06

Introduction

India harbours a diverse range of mammals, including deer and antelope species (Prasad and Prabhakar, 2020; Prasad and Ahmed, 2021)**.** Among them, *Antilope cervicapra*, commonly known as the Blackbuck, is a threatened antelope species found in the Indian subcontinent (Arandhara *et al.,* 2021). Blackbucks face challenges such as habitat destruction and increased human activity, jeopardizing their survival in the wild (Das *et al.,* 2018; Gyawali *et al.,* 2020). They exhibit gregarious behaviour and are preyed upon by carnivores like wolves and jackals. The species is legally protected under the Wildlife (Protection) Act, 1972, categorized as of 'Least Concern' (LC) by IUCN, and listed in Appendix II of CITES. However, their population has declined significantly, with small, isolated, and fragmented patches remaining in various states, including Haryana (Delu *et al.,* 2022; 2023). In the past, blackbucks roamed freely throughout Haryana, with substantial numbers reported, but their distribution has reduced drastically. In recent decades, habitat loss, poaching, loss of true grassland due to agricultural intensification along with the newly emerged predator, *i.e.,* feral dogs, are the main reasons for the reduced population size of blackbuck in Haryana especially in its western part (Delu *et al.,* 2021).

Modern science requires a molecular approach to assess genetic diversity for various purposes, including conservation and understanding evolutionary relationships (Markov *et al.,* 2015). Molecular studies have been used for genetic diversity analysis, zoogeography, and evolutionary phylogeny (Shukla *et al.,* 2019; Jana and Karanth, 2022). The mitochondrial molecular markers have been used to study a comparative assessment of phylogeny of *A. cervicapra* with its implications on biogeography and taxonomy of Indian antelopes. The amplification of even short region of COI and Cytb amplified from fecal samples can efficiently identify the species under consideration and has wide potential in forensics and conservation programmes. The utility of molecular phylogenetics in resolving evolutionary

[1]Department of Zoology & Aquaculture, CCS Haryana Agricultural University, Hisar-125004, Haryana, India

[2]Department of Animal Biotechnology, Lala Lalpat Rai University of Veterinary and Animal Sciences, Hisar-125004, Haryana, India

[3]Wildlife Institute of India, Dehradun-248001, Uttarakhand, India

Corresponding Author: Dr. Kanisht Batra, Department of Animal Biotechnology, Lala Lalpat Rai University of Veterinary and Animal Sciences, Hisar-125004, Haryana, India e-mail: drkanishtbatra@gmail.com

How to cite this article: Delu, V., Singh, D., Batra, K., Maan, S., & Saini, D. (2024). Genetic Diversity of Small, Isolated and Fragmented Blackbuck (*Antilope cervicapra*, L.) Populations of Haryana based on mtDNA Barcoding. Ind J Vet Sci and Biotech. 20(5), 32-37.

Source of support: Nil

Conflict of interest: None

Submitted 15/05/2024 **Accepted** 24/06/2024 **Published** 10/09/2024

relationships between organisms has been demonstrated as a crucial tool (Singh *et al.,* 2023). Therefore, this study was aimed to analyze the genetic diversity of Blackbuck populations in Haryana, India, using mtDNA COI gene. The focus was on several isolated and fragmented populations of Blackbuck inhabiting the semi-arid region of Haryana.

Materials and Methods

Collection of Samples

The study was conducted at LUVAS, Hisar (Haryana) during the period from June 2020 to July 2021. Eleven fecal pellets of Blackbuck population (morphological basis) were collected from the agricultural fields of Haryana, and their surroundings, not older than 72 h when animals were absent from collection site. Samples were collected using sterilized dry forceps and placed inside collection tube which contained either 95% of ethanol or silica gel subject to the conditions of the samples (fresh or dried) or in a clean and dry zip lock pouch. These Blackbuck habitats are located in the biogeographic province category 4-A of Semi-arid habitat in Punjab Plains, and fall under the 'Trans-Gangetic Plains Regions' agroclimatic zone, which is part of the 'Arid to Semi-arid' climatic zone. This region is known for its low rainfall, arid conditions, and extreme temperatures. The collection tube and zip lock pouch were placed in an ice box and immediately transported to the laboratory at ABT, LUVAS, Hisar. Subsequently, the samples were preserved at a temperature of -20°C until required for future analysis. In addition, total six tissue samples were collected from the post-mortem section of the department of Veterinary Pathology of the institute. These samples were collected in collection tubes in the presence of veterinary experts at the time of post-mortem of the animal and stored at -80 °C till further use.

Extraction of Genomic DNA

The total genomic DNA was extracted using conventional DNA extraction method, namely the Phenol: Chloroform: Isoamyl alcohol (PCI) method and the Zymo Quick-DNA™ Kit (Murphy Ave., Irvine, CA) with some modifications and standardization of the DNA extraction protocols (Sambrook *et al.,*1989), The concentration of DNA was measured with Nanodrop instrument (Thermo scientific) and samples with an OD ratio between 1.7-1.9 were deemed to have acceptable purity and were utilized in subsequent experiments.

Designing of PCR Primers

By utilizing the Primer 3 v 0.4.0 software, we designed specific primers for amplifying a partial region (438 bp) of the mitochondrial cytochrome c oxidase subunit 1 gene in Blackbucks. These primers were designed to target a specific conserved part of cytochrome c oxidase subunit 1 gene for amplification. The selected primer pairs used in this study were (1F: 5'-TGGAATCGTCCTCGCTAACT-3' and 1R: 5'-GGAGGGCATCCATTTAGTCA-3') which yielded a product size of 438 bp

PCR Amplification, Product Purification and Sequencing

The amplification of the cytochrome c oxidase subunit 1 (COI) region was performed using various conserved primer sequences. The concentration of each primer (ranging from 5 pmole to 20 pmole) was optimised using checker board titration. The thermal amplifications were optimized as initial denaturation at 98°C for 30 sec, 40 cycles of denaturation at 95°C for 10 sec, annealing and extension at 50°C and 72°C for 30 sec with a gradient of 2°C. The agarose gels electrophoresis was carried out for better resolution of DNA products (438 bp) amplified using PCR. Desired bands were cut and Gel Extraction Kit® was done with some modifications of the standard protocols for better purity. The Capillary DNA sequencing was performed utilizing the chain termination method on an automated DNA first generation Sanger sequencer.

Genetic Diversity and Phylogenetic Data Interpretation

The total seventeen samples, (six tissues and eleven fecal) were processed, and their DNA sequences, which were obtained in both forward and reverse directions through sequencing, were aligned and analyzed using Sequencer version 4.9, developed by Gene Codes Corporation in Ann Arbor, MI, USA. The sequences were aligned separately using the Clustal X 1.8 multiple alignment program, and the resulting alignments were visually inspected. The nucleotide diversity (p), haplotype diversity (h), and polymorphic sites (s) were analyzed using Dna SP 5.050. The average pair-wise genetic differences among populations were estimated by utilizing the MEGA software (Kumar *et al.,* 2018). The Tamura-3 parameter with the discrete gamma distribution (TN92+G), which had the lowest Bayesian information criterion (BIC) score, was employed to calculate the genetic distance. Phylogenetic evaluations were carried out using Beast version 1.752. Mismatch distribution plots were generated using Dna SP 5.050 v (Librado and Rozas, 2009) to determine whether the Blackbuck populations were in demographic equilibrium or had undergone a bottleneck Tajima's D57, Fus Fs58, and Fu, and Lis F and D59 were used to assess the neutrality of the sequences based on various statistical approaches for short DNA sequences, using Dna SP 5.050.

The Tajima's D and Fu's Fs tests are generally used to evaluate the demographic equilibrium of the population, with negative and positive values indicating significant departures from equilibrium. To estimate population genetic divergence, the corrected average pair-wise differences were calculated using MEGA X (1) with 1000 bootstrap replications, and genetic distances were determined by utilising the p distance models as well as the Kimura 2-parameter (K2P)

model. In addition, MEGA's Kimura-2 parameter distance was used to calculate pair-wise evolutionary sequence divergence among populations.

Results and Discussion

DNA Barcoding techniques using mitochondrial genes revealed its vast application in species identifications and forensic aspects especially to check illegal wildlife trafficking. The present study also successfully identified the species from tissues as well as faecal samples using forensically important nucleotide sequences (FINS). The genetic diversity among Haryana Blackbuck species from different locations has earlier also shown maximum homology with *A. cervicapra* with the sequences of NCBI data (Kumar *et al.,* 2017). They used a different region of mt.cytochrome c oxidase unit 1 gene for primer designing and amplification. The present study also relates with the earlier findings but in a different context because the region being used for primer designing and amplification was present at different loci in mitochondrial genome. Total 17 samples of different populations of western Haryana were screened for molecular diagnostic of the species through DNA Barcoding and to reveal the species genetic diversity and other parameters by using the partially amplification of mt.cyt. C oxidase subunit 1 gene. Samples from Badopal Area, Fatehabad (Lat: 29.4168533; Long: 75.5796286) represent a single isolated and fragmented population surrounded with high human habitation and intensive agriculture. Samples denoted with Mangali, Hisar (Lat: 29.0203125; Long: 75.6942611) were also a semi-arid population of Blackbuck and fragmented in nature and the tissue samples from Deer Park Hisar (Hisar Division) (Lat: 29.179791; Long: 75.5212055) and CCS HAU Hisar (Lat: 29.1503508; Long: 75.7057018) were the parts of different populations in Hisar division region not from Badopal and Mangali area population. Behaviourally, there was a negligible migration between populations in recent decades due to agricultural intensification and human habitation in those areas. Good quality of DNA (5-50 µg/µL) was extracted from samples and best amplified at the annealing temperature of 50°C (Fig. 1).

The genetic p-distance between Badopal and Mangali was 0.6% followed by the Badopal and Deer Park Hisar 0.3%, and between Badopal and Other Region 1.6%. Similarly, the distances between Mangali and Deer Park Hisar and between Mangali and Other Region was 0.4% and 1.7%, respectively, while the p-distance between Deer Park Hisar and Other Region was 1.3%. This data highlights the genetic variation among the Blackbuck populations across different locations. This indicated high genetic differentiation. A low genetic distance was observed for Deer Park Hisar with Badopal 0.3%. However, high genetic distance of 1.7% was observed between Mangali and Other Region populations.

Genetic diversity estimates, *i.e.,* haplotype diversity (Hd) and nucleotide diversity (π) indices were relatively low in Haryana Deer park (Hd 0.33; π 0.0012) in comparison to Badopal (Hd 0.83; π 0.0057) and other populations across its range (Table 1). In the haplotypes network, all these samples were spanned haplotypes concerning their geographic locality and indicated sequential mutation. The overall haplotype diversity (Hd 0.65) and nucleotide diversity (π 0.013) values show the population of Blackbuck may have moderate diversity. Further, neutrality test values (Table 2), indicated an excess of rare nucleotide site variants under a neutral model of evolution. Here, the overall population of Blackbuck showed negative Tajima D and it was statistically

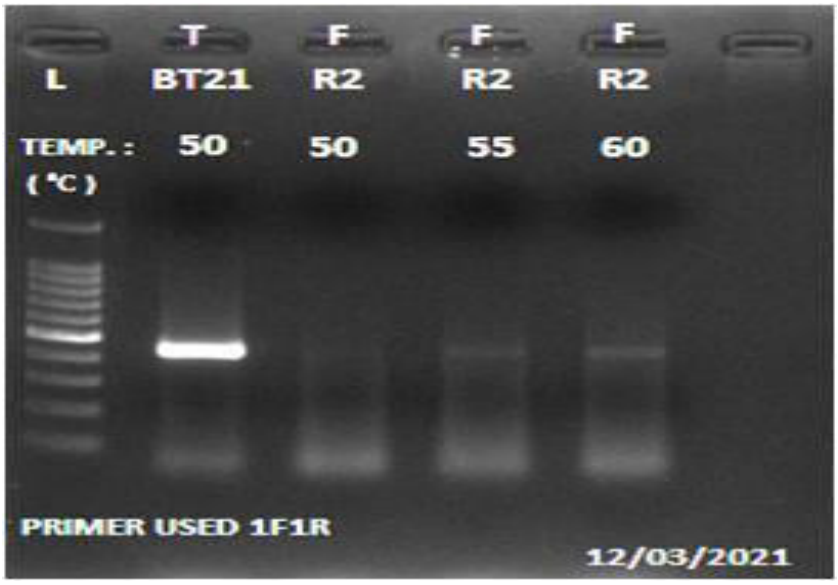

Fig. 1: Agarose gel electrophoreses of faecal and tissue samples showing product size of 438 bp. Lane 1: Ladder, Lane 2: Tissue samples, Lane 3-5: Faecal samples, Lane 6: Non template control

not significant for all populations. The statistically non-significant positive Fu's Fs values were observed in all Blackbuck populations, which showed that there is an excess of rare mutations and can be taken as evidence of population stability and COI analysis also indicated the population of blackbuck was stable and equilibrium state.

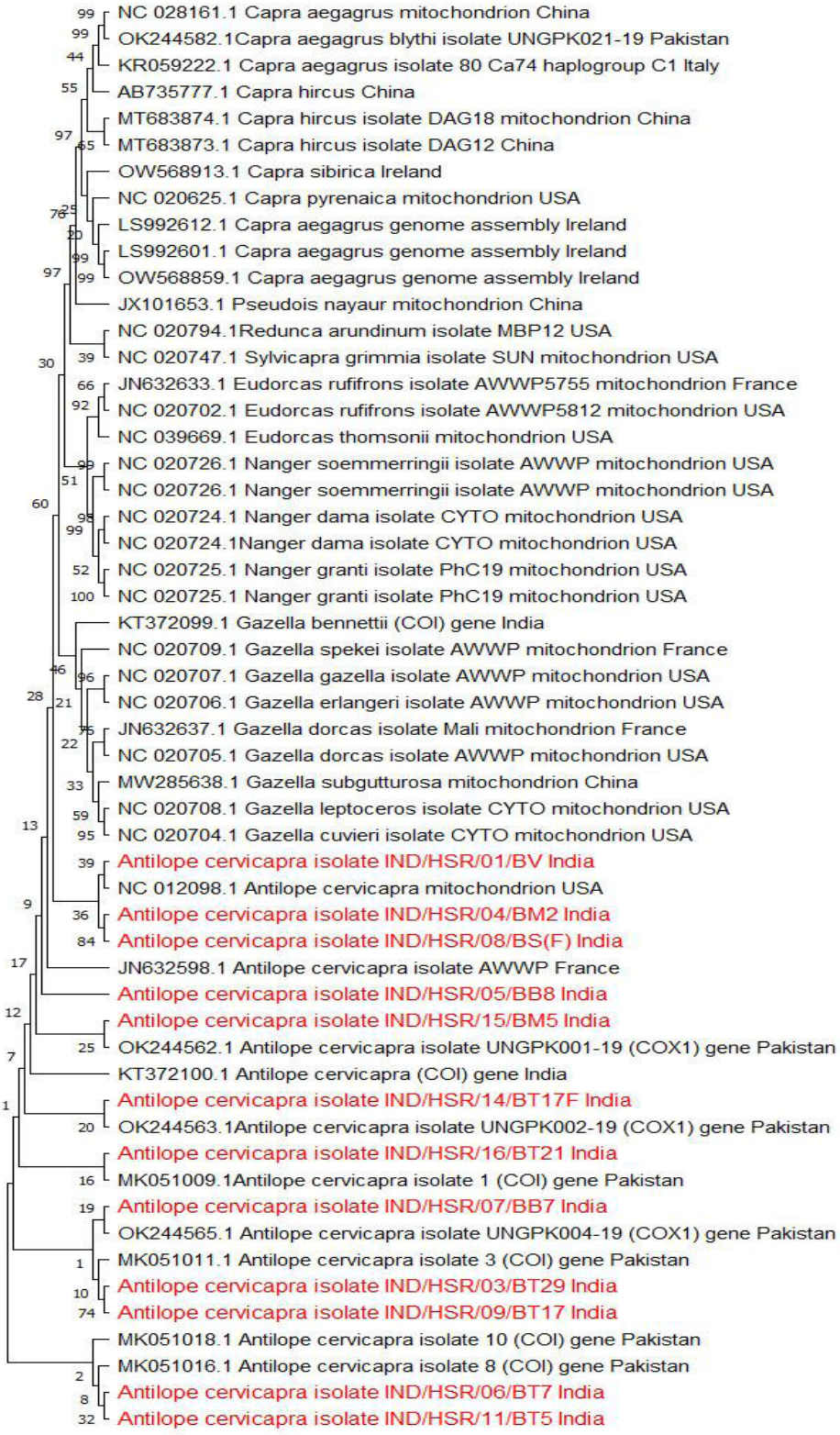

Fig. 2: Phylogenetic analysis of selected blackbuck populations based on COI amplification and the database of this gene available at NCBI for this species

Table 1: mtDNA diversity indices of all selected populations (n= 33)

Site Name	Sample size	Segregating sites (S)	Haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity (π)
Badopal, Haryana	9	7	6	0.83	0.0057
Deer park	6	1	2	0.33	0.0012
Mangali	2	2	2	1.00	0.0073
Other region (NCBI data bank)	16	28	5	0.45	0.0216
Total	**33**	**37**	**12**	**0.65**	**0.0131**

The COI sequences of Blackbuck populations available in the NCBI database and sequences from the present investigation were used to generate the phylogenetic tree. The phylogenetic results between the populations of Badopal, Deer Park Hisar, Mangali and other region indicated the close genetic relationship among Badopal, Deer Park Hisar and Mangali (Fig. 2). Similarly, the mitochondrial affiliation of Badopal, Deer Park Hisar, Mangali with other Region was close. Additionally, the study utilized Cyt c-based genetic distance and phylogenetic tree analyses to reveal that the Blackbuck population is undergoing migration. This finding indicated that population structure may be resolved by examining populations from Badopal, Deer Park Hisar, and Mangali, as these populations are found in the clade of Blackbuck species with mixed populations and need for maintaining connectivity among the populations. Populations in Badapol and Mangali were almost identical and Badopal population also closely related to JN632598.1, MK051009, MK051010, MK051018, and MK051018.1 (MK Series Lahore Pakistan). Deer Park Hisar show close relationship with MK051016, MK051013, MK051014, KT372100 and MK051019. The out-group exhibits a distinct clade, indicating that the maternal genetic analysis reveals a mixed inheritance pattern among Blackbuck populations from various locations. The data generated in this study will serve as crucial baseline information for genetic monitoring and evolutionary relationship analysis. The study conducted on Blackbuck samples from the Pakistan region, revealed low genetic variations within *A. cervicapra* and significant genetic divergence between Blackbuck and other species (Abbas *et al.,* 2020). The present study corroborated these results, showing minimal genetic variations (99% identical) among all screened samples and significant genetic divergence with related species. The populations of *A. cervicapra* under study have a close relationship with (98-99%) identity with Pakistan population and (95%) with species from other regions of the world.

Table 2: Fu's Fs test and Tajima's D result

	Fu's Fs test	Tajima's D
Badopal	-2.311	-1.4861
Mangali	0	0
Deer park Hisar	-0.003	-0.9330
Other region	±3.856	-1.3523

In general, the use of DNA barcodes serves as a fundamental reference point for assessing genetic diversity. As such, knowledge of the degree of genetic variation is an essential prerequisite for devising effective conservation strategies. Moreover, such information represents a critical resource for addressing issues surrounding species identification and wildlife-related criminal activities in wildlife forensic contexts.

Conclusion

This study specifically employed mtDNA analysis to explore the genetic diversity and phylogenetics of small Blackbuck populations in Haryana, India, using tissue and fecal samples amplified with the cytochrome c oxidase subunit I gene. These findings also shed light on the genetic makeup of the studied populations, emphasizing the importance of maintaining connectivity among fragmented population.

Acknowledgements

The authors are thankful to the Department of Animal Biotechnology, College of Veterinary and Animal Sciences, LUVAS and Department of Zoology & Aquaculture, CCS HAU, Hisar, for providing financial, technical, logistic support and human resource for the smooth conduction of work. The authors are also grateful for the then DFO Hisar and PCCF cum Chief Wildlife Warden (CWW) Haryana Forest Department for granting the permission of sample collection.

References

Abbas, G., Mustafa, S.A., Sajjad, S., Sultana, S., Shahzad, K., & Jabeen, R. (2020). Mitochondrial D-loop sequencing reveals genetic diversity and structure of the blackbuck (*Antilope cervicapra*) populations in Pakistan. *Journal of Animal and Plant Sciences, 30*(2), 726-734.

Arandhara, S., Sathishkumar, S., Gupta, S., & Baskaran, N. (2021). Influence of invasive *Prosopis juliflora* on the distribution and ecology of native blackbuck in protected areas of Tamil Nadu, India. *European Journal of Wildlife Research, 67*(3), 1-131.

Das, U.K., Kar, S., & Pattnaik, S.K. (2018). Forage and feeding ecology of Indian antelope or blackbuck (*Antilope cervicapra* Linn 1780) in Ganjam district, south Odisha, eastern India. *International Educational Applied Research Journal, 2*(11), 1-15.

Delu, V., Singh, D., Dookia, S., Priya, G., Godara, A., & Karwasra, V. (2023). An insight into population structure and seasonal herd pattern of blackbuck *Antilope cervicapra* (Linnaeus,

1758) (Mammalia: Artiodactyla: Bovidae) in semi-arid region of western Haryana, India. *Tropical Ecology,* 11.

Delu, V., Singh, D., & Dookia, S. (2022). Who lets the dog out? stray dogs emerging as a potential threat for blackbuck and other wildlife in western Haryana, India: A preliminary assessment. *Ambient Science 9*(2), 01-4.

Delu, V., Singh, D., Dookia, S., & and Priya, K. (2021). Seasonal food preferences and group activity pattern of Blackbuck *Antilope cervicapra* (L., 1758) (Mammalia: Cetartodactyla: Bovidae) in a semi-arid region of western Haryana, India. *Journal of Threatened Taxa, 13,* 19937-19947.

Gyawali, U., Mandal, R.A., Mathema, A., & Subedi, A. (2020). Assessing the population dynamics of blackbuck, its habitat condition and people interaction in blackbuck conservation area, Khairapur, Nepal. *Annals of Carnegie Museum, 86*(3), 215-228.

Jana, A., & Karanth, P.K. (2022). Genetics reveals origin and evolution of blackbuck, chinkara and their close relatives. *Molecular Phylogenetics and Evolution, 166,* 1072521.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547-1549.

Kumar, V., Sharma, N., & Sharma, A. (2017). DNA barcoding of the Indian blackbuck (*Antilope cervicapra*) and their correlation with other closely related species. *Egyptian Journal of Forensic Sciences, 7*(1), 31

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*(11), 1451-1452.

Markov, G.G., Kuznetsova, M.V., Danilkin, A.A., Kholodova, M.V., Sugár, L., & Heltai, M. (2015). Genetic diversity of the red deer (*Cervuselphus* L.) in Hungary revealed by cytochrome b gene. *Acta Zoologica Bulgarica, 67,* 11-17.

Prasad, S., & Ahmed, R. (2021). Report of an elegant species (*Antilope cervicapra* Linn.) in non-protected area of Shahabad, Bihar, India. *Journal on New Biological Reports*, *10*(1), 31-37.

Prasad, S., & Prabhakar, C.S. (2020). Docility behavioral development in nilgai (*Boselaphustragocamelus*), a sign of taming towards domestication. *Current Journal of Applied Science and Technology, 39*(41), 30-39.

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* 2nd ed., Cold Spring, Harbor Laboratory Press.

Shukla, M.A., Joshi, B.D., Kumar, V.P., Mehta, A.K., & Goyal, S.P. (2019). Investigating the genetic diversity and presence of forensically informative nucleotide sequences in Indian antelope (*Antilope cervicapra*) using multiple genes of the mitochondrial genome. *Molecular Biology Reports, 46*(6), 6187-6195.

Singh, V.K., Joshi, B.D., Dalui, S., Ghosh, A., & Jabin, G. (2023). Genetic diversity and population structure of Himalayan tahr (*Hemitragus jemlahicus*) from Western Himalaya. *Mammalia, 83*(3), 238-244.