Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur)* in Himalayan Region

Thesis

submitted to the

Forest Research Institute (Deemed to be University),
Dehradun, Uttarakhand

For the award of the Degree of

Doctor of Philosophy

(Forest Biotechnology)

By

Deepesh Saini

Registration No: 19Ph.D593

Under the supervision of

Dr. K.Vishnupriya
Scientist E & Supervisor

Wildlife Institute of India, Dehradun, Uttarakhand

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

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Wildlife Institute of India

DECLARATION

I hereby declare that the thesis entitled **"Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur*) in Himalayan Region"** is an original piece of research conducted out by me under the supervision of Dr. K. Vishnupriya, Scientist-E, Wildlife Institute of India, and co-supervision of Dr. S. K. Gupta, Scientist-F, and Dr. S. Sathyakumar, Former Scientist-G, Wildlife Institute of India. This thesis has been submitted to the **Forest Research Institute (Deemed to be University), Dehradun**, for the award of the degree of **Doctor of Philosophy in Forest Biotechnology** and has not been submitted elsewhere for the award of any other degree or diploma. The work embodied in this thesis is my own and contributes to the advancement of knowledge on the subject.

Place: Dehradun

Date: 29/12/2025

Deepesh

(Deepesh Saini)

Animal Ecology & Conservation Biology

पत्रपेटी सं. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स: 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

Dr. K. Vishnupriya Ph.D

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

CERTIFICATE

This is to certify that the thesis **titled "Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur)* in Himalayan Region "** Submitted by Mr. Deepesh Saini (Registration No. 19PHD593) to Forest Research Institute (Deemed to be) University (FRI), Dehradun, for the award of the degree of Doctor of Philosophy in Forestry (Forest Biotechnology) is a record of bonafide research work carried out by him, under my supervision. No part of this thesis has been submitted for any other degree/diploma of the same institution where the work is carried out or to any other institution. It fulfils all the requirements of the ordinance governing the award of a Ph.D. Degree of FRI, Deemed to be University, Dehradun. Mr. Deepesh Saini has adequate attendance during his thesis work and he was not engaged in any paid assignment.

Place - Dehradun
Date - 29/12/2025

K. Vishnupriya

Dr K. Vishnupriya
(Supervisor)
Wildlife Institute of India

Vishnupriya Kolipakam
Ph.D., Faculty, Dept. of Animal
Ecology & Conservation Biology
Wildlife Institute of India, Dehradun

पत्रपेटी सं. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स : 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

Dr. S.K. Gupta Ph.D.

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

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Place - Dehradun
Date - 29/12/2025

Dr S.K. Gupta
(Scientist F & Co-Supervisor)
Wildlife Institute of India

पत्रपेटी सं. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स: 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

Dr. S. Sathyakumar Ph.D.

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

CERTIFICATE

This is to certify that the thesis **titled "Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur)* in Himalayan Region "** Submitted by Mr. Deepesh Saini (Registration No. 19PHD593) to Forest Research Institute (Deemed to be) University (FRI), Dehradun, for the award of the degree of Doctor of Philosophy in Forestry (Forest Biotechnology) is a record of bonafide research work carried out by him, under my supervision. No part of this thesis has been submitted for any other degree/diploma of the same institution where the work is carried out or to any other institution. It fulfils all the requirements of the ordinance governing the award of a Ph.D. Degree of FRI, Deemed to be University, Dehradun. Mr. Deepesh Saini has adequate attendance during his thesis work and he was not engaged in any paid assignment.

Place - Dehradun
Date - 29/12/2025

Dr S. Sathyakumar
(Former Scientist G & Co-Supervisor)
Wildlife Institute of India

पत्रपेटी सं. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स: 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

Forest Research Institute (Deemed to be) University Dehradun

This is to certify that **Mr. Deepesh Saini** (Enrolment no 19PHD593) carried out research work under **Dr. K. Vishnupriya** (Scientist E), and of Co-Supervision **Dr S. K. Gupta** (Scientist F), and **Dr S. Sathyakumar**, (Former Scientist G) of Wildlife Institute of India, Dehradun.The topic of the research registered with FRI (Deemed to be) University **"Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur)* in Himalayan Region "**.The scholar presented his work in the pre thesis submission seminar held on 04th July 2025 and the RAC found the work to be satisfactory and approve the work to be presented in the form of thesis for evaluation for "Award of Ph.D Degree" by FRI (Deemed to be) University, Dehradun.

Dr. K. Vishnupriya
Scientist E & Supervisor
Wildlife Institute of India

Dr. S.K. Gupta
Scientist F & Co - Supervisor
Wildlife Institute of India

Dr. S. Sathyakumar
Former Scientist G & Co-Supervisor
Wildlife Institute of India

Dr. Samrat Mondol
Scientist F & Nodal officer
Member Secretary
Wildlife Institute of India

Dr. Gopi. G.V.
Scientist F & Member
Wildlife Institute of India

Dr. G. Talukdar
Scientist F & Member
Wildlife Institute of India

Dr. Amit Kumar
Scientist E & Member
Wildlife Institute of India

Dr. Ruchi Badola
Scientist G & Dean, FWS
Chairman, RAC
Wildlife Institute of India

Dr. S. Dutta
Scientist E & Member
Wildlife Institute of India

पत्रपेटी स. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स : 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

Minutes of meeting of RAC for Pre-thesis submission seminar

Name of the Scholar	Deepesh Saini
Registration Number	19PHD593 01/03/2020
Name of Discipline & Topic of Research	Forest Biotechnology "Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur)* in Himalayan Region"
Name of Supervisor	Dr. K. Vishnupriya, Scientist E
Name of Co-Supervisor	Dr S. K. Gupta, Scientist F Dr S. Sathyakumar, Former Scientist G
Name of Research Centre/ Institute	Wildlife Institute of India
Date of meeting of RAC	04/07/2025

Remarks if any of the supervisors:

SATISFACTORY

K. Vishnupriya

Remarks if Chairman/ Expert Members of R.A.C.:

Dr. K. Vishnupriya
Scientist E & Supervisor
Wildlife Institute of India

Dr S. K. Gupta
Scientist F & Co - Supervisor
Wildlife Institute of India

Dr. Samrat Mondol
Scientist F & Nodal officer
Member Secretary
Wildlife Institute of India

Dr S. Sathyakumar,
Former Scientist G & Co-Supervisor
Wildlife Institute of India

Dr. Gopi. G.V.
Scientist F & Member
Wildlife Institute of India

Dr. G. Talukdar
Scientist F & Member
Wildlife Institute of India

Dr. Amit Kumar
Scientist D & Member
Wildlife Institute of India

Dr. S. Dutta
Scientist E & Member
Wildlife Institute of India

Dr. Ruchi Badola
Scientist G & Dean, FWS
Chairman, RAC
Wildlife Institute of India

संकायाध्यक्ष / Dean
भारतीय वन्यजीव संस्थान
WILDLIFE INSTITUTE OF INDIA
देहरादून / Dehradun

Minutes of the Pre thesis seminar

The following comments were received in the pre thesis held on 04/07/2025 at Wildlife Institute of India, Dehradun. All the given comments were incorporated in the thesis.

Comments	Responses
Did you see any different in protein coding gene between western and eastern Himalaya	The comments are incorporated in the thesis- (Till now "Till now, no change has been observed in protein-coding genes, as the detected variations are synonymous (silent) substitutions")
Show distribution map Where is western and eastern area	The comments are incorporated in the thesis-
In Limitation Nepal part should also be mentioned that it is not sampled	The comments are incorporated in the thesis- (Samples from Nepal could not be included in the present study due to the absence of research & collection permits from the concerned authorities.

सं./No. 615 /19PHD593/2019/एफ़आरआईडीयू
वन अनुसंधान संस्थान सम विश्वविद्यालय
पी. ओ. आई.पी.ई. कौलागढ़ रोड, देहरादून -248 195

दिनांक: 23-12-2020
ई-मेल: registrarfriu@icfre.org

सेवा में,

श्री दीपेश सैनी,
द्वारा डॉ. के. विष्णुप्रिया,
वैज्ञानिक-सी,
भारतीय वन्यजीव संस्थान,
पत्रपेटी स 18, चंद्रबानी, देहरादून-248001

विषय: वानिकी में डॉक्टर ऑफ फिलॉसफी डिग्री के लिए पंजीकरण।

प्रिय महोदय,

आपको अवगत करना है कि इस संस्थान में वानिकी में डॉक्टर ऑफ फिलॉसफी की डिग्री के लिए शोध छात्र (रिसर्च स्कॉलर) के रूप में आपके नामांकन हेतु निम्नलिखित निर्णय लिए गए हैं:

1. आपको डॉक्टर ऑफ फिलॉसफी के लिए दिनांक **01.03.2020** से **31.08.2025** तक पीएचडी शोध छात्र के रूप में पंजीकृत किया गया है।
2. आपका नामांकन संख्या है : **19PHD593**
   (सभी प्रकार के पत्राचार में इस नामांकन संख्या का उल्लेख करें)
3. अनुसंधान केंद्र का नाम: **भारतीय वन्यजीव संस्थान, देहरादून**
4. वन अनुसंधान संस्थान सम विश्वविद्यालय द्वारा स्वीकृत विषय **"Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur*) in Himalayan Region."**
5. शाखा का नाम: **Forest Biotechnology**
   (पीएचडी अध्यादेश के खंड 3.3 के अनुसार)
6. (i) पर्यवेक्षक का नाम: **Dr. K. Vishnupriya**
   (ii) सह पर्यवेक्षक का नाम: **Dr. S.K. Gupta**
   Dr. S. Sathya Kumar
7. आपको निम्न शुल्क जमा करने की सलाह दी जाती हैं:-

(अ) प्रयोगशाला शुल्क की अगली किस्त संबंधित एफ़आरआई सम विश्वविद्यालय/अनुसंधान केंद्र पर बैंक ड्राफ्ट के जरिए देय है।

(आ) शोधप्रबंध जमा करने तक पंजीकरण के प्रत्येक वर्ष हेतु मार्च माह में संबंधित एफ़आरआई सम विश्वविद्यालय/शोध केंद्र में पुस्तकालय शुल्क देय होगा।

(इ) शोधप्रबंध जमा करने तक प्रत्येक वर्ष के लिए एफ़आरआई सम विश्वविद्यालय में पंजीकरण अवधि दौरान मार्च महीने में देय वार्षिक शुल्क देय होगा।

(ई) उपरोक्त शुल्क नियत महीने के दौरान अर्थात प्रत्येक वर्ष मार्च माह में इस कार्यालय में जमा करना होगा अन्यथा विलंब होने पर रु. 1000/- (बैंक ड्राफ्ट) विलंब शुल्क भी जमा करना होगा।

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8. The research scholar is required to submit the six monthly progress report till the wo presented in the pre-thesis submission seminar and is approved by the Research Advis Committee for submission of thesis.

9. The research scholar shall appear before the Research Advisory Committee to make a presentation of the progress of his/her work for evaluation and further guidance.

10. Ph.D. Scholar shall be required to be present in the research center concerned for a minimum period of two years from the date of registration. Their presence shall be duly recorded and maintained in the research center concerned.

11. **Registration of a Ph.D. Scholar is liable to be cancelled by the Director at any time if:-**

i. Two consecutive six monthly progress reports are not submitted at all or are not satisfactory as per recommendations/comments of RAC.
However, the research scholar is required to submit the 1st six monthly progress report through his/her Supervisor & Chairman of RAC and 2nd and 3rd six monthly progress reports duly reviewed by RAC. The candidate will make six monthly presentation of 2nd & 3rd six monthly Progress report before RAC. After that all the 6 monthly Progress Reports shall be submitted through supervisor & Chairman, RAC while annual presentation would be held before RAC.
The six monthly progress reports are to be submitted till pre-thesis submission seminar.

ii. The attendance of Research Scholar is less than 75% in any term.

iii. The scholar violates the clause 5.1.4 of the PhD ordinance regarding compulsion of 2 years Study leave for pursuing PhD in case of In-service candidates (except the employees of ICFRE and Research Centers of FRI Deemed to be University).

12. No Ph.D. Scholar (except in-service candidates availing study leave) shall accept during the period of research any paid assignment apart from Research Fellowships, Research Assistantship etc. (in the same institute) unless in the opinion of the RAC such an assignment will not interfere with his/her research work.

13. A Ph.D. Scholar shall not be permitted to take any other degree course, but may be permitted by the RAC to take part-time Diploma or Certificate course(s) not affecting the scholars research work adversely.

14. **A Research Scholar is required to pursue research in the Institute/Research Centre under the Supervisor on the approved subject for not less than twenty-four months commencing from the date of his/her registration.**

15. **The Research Scholar may not later than three months from the date of issue of registration letter, modify the scheme of the research work or nature or scope of the subject, on the recommendation of the Supervisor and RAC, with the approval of Director.**

16. In case a Research Scholar does not submit his/her thesis within a period of 6 years from the date of his/her admission unless the term is extended by the Research Degree Committee on the specific recommendation of the Research Advisory Committee for a period of upto1 calendar year, his/her registration shall lapse.

The recommendations of the R.A.Cs for extension of term of registration must reach this office before expiry of the term of registration.

The women candidates and Persons with Disability (more than 40% disability) may be allowed a relaxation of two years in the maximum duration of registration i.e. 5 years and six months. In addition, during the entire period of registration the women candidates may be provided Maternity Leave/Child Care Leave once in the entire duration of Ph.D. for up to 240 days with the approval of Vice Chancellor on the recom mendation of Supervisor/Head of Division/Nodal Officer of the Research Centre concerned.

17. Prior to the submission of the thesis but at least 3 months before the expiry of term of registration, the scholar shall make a presentation in the Department before the Research Advisory Committee of the Institution concerned in Pre-thesis Submission Seminar. The minutes of RAC meeting for pre-thesis submission seminarts to be send to the Registrar, FRI Deemed University with full comments alongwith a panel of examiners duly signed by R.A.C.

18. Ph.D. scholars must publish at least one (1) research paper in refereed journal and make two paper presentations in conferences/seminars before the submission of the thesis for adjudication, and produce evidence for the same in the form of presentation certificates and/or reprints. While submitting for evaluation, the thesis shall have an undertaking from the research scholar and a certificate from the Research Supervisor attesting to the originality of the work, vouching that there is no plagiarism and that the work has not been submitted for the award of any other degree/diploma of the same Institution where the work was carried out, or to any other Institution..

19. **Please ensure that the clause 13 of the Ph.D. Ordinance is fully complied with before submission of the thesis to University.**

20. **Please note that your Registration as Research Scholar is to be governed as per rules, regulation, and ordinances of FRI Deemed to be University, with applicable amendments made by the University from time to time. For all further correspondence, please quote your enrolment number.**

(ए.के. त्रिपाठी)
कुलसचिव
व.अ.सं सम विश्वविद्यालय

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4. डॉ वी.पी. उनियाल, (नोडल अधिकारी एफ़आरआईडीयू) भारतीय वन्यजीव संस्थान, पत्रपेटी स 18, चंद्रबानी, देहरादून-248001

(ए.के. त्रिपाठी)
कुलसचिव
व.अ.सं सम विश्वविद्यालय

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

CERTIFICATE OF PLAGIARISM CHECK

Name of the research scholar	Deepesh Saini
Title of the thesis	Molecular Phylogeny and Population Genetics of Himalayan Blue Sheep (*Pseudois nayaur*) in Himalayan Region
Name of the supervisor	Dr. K. Vishnupriya, Scientist E
Name of the Co-supervisors	Dr S. K. Gupta, Scientist F Dr S. Sathyakumar, Former Scientist G
Department/Institution/Research Centre	Wildlife Institute of India Dehradun, Uttarakhand
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Name and Signature of the researcher	Deepesh Saini
Name and Signature of the supervisor	Vishnupriya Kolipakam Ph.D., Faculty, Dept. of Animal Ecology & Conservation Biology Wildlife Institute of India, Dehradun

पत्रपेटी सं. 18, चन्द्रबनी, देहरादून-248 001, उत्तराखण्ड, भारत
P.B. No. 18, Chandrabani, Dehradun-248 001, Uttarakhand, INDIA
ई.पी.ए.बी.एक्स. :+91-135-2640114, 2640115, 2646100, फैक्स : 0135-2640117
EPABX : +91-135-2640114, 2640115, 2646100, Fax : 0135-2640117
ई–मेल/E-mail : wii@wii.gov.in, वेब/website : www.wii.gov.in

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Submission Information

Author Name	Deepesh
Title	Thesis
Paper/Submission ID	4253861
Submitted by	manohar@wii.gov.in
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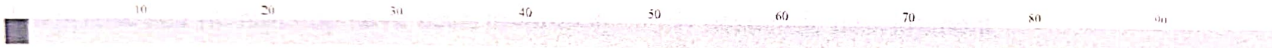

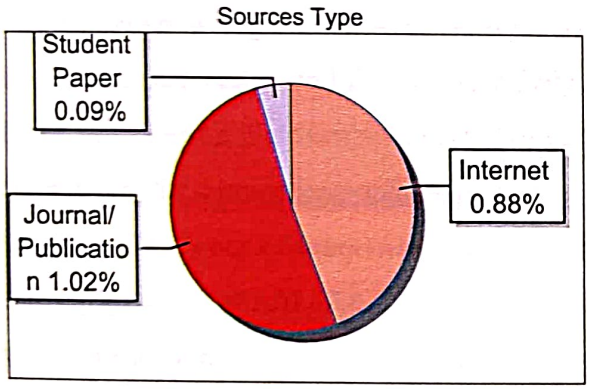

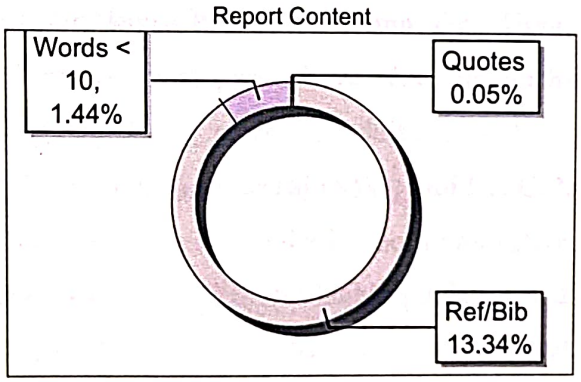

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LIST OF ABBREVIATIONS

Abbreviation	Full Forms
Genetic And Molecular Biology	
Dna	Deoxyribonucleic Acid
Mtdna	Mitochondrial Dna
Gdna	Genomic Dna
Rna	Ribonucleic Acid
Snp	Single Nucleotide Polymorphism
Ssr	Simple Sequence Repeat
Cyt B	Cytochrome B Gene
Pcr	Polymerase Chain Reaction
Mpss	Massively Parallel Signature Sequencing
Ffpe	Formalin-Fixed Paraffin-Embedded
Fnac	Fine Needle Aspiration Cytology
Genetic Diversity & Population Structure	
Ho	Observed Heterozygosity
He	Expected Heterozygosity
H / Hd	Haplotype Diversity
Π	Nucleotide Diversity
Ar	Allelic Richness
Na	Number Of Alleles

Ne	Effective Number Of Alleles
F	Fixation Factor
I	Shannon's Information Index
Pic	Polymorphic Information Content
K	Genetic Clusters
Evolution & Phylogenetics	
Mj	Median Joining (Network)
Mcmc	Markov Chain Monte Carlo
Fu / Fs	Fu's Fs Statistic
Esu	Evolutionary Significant Units
Mu	Management Units
Amova	Analysis Of Molecular Variance
Statistical Tools & Analysis	
Dapc	Discriminant Analysis Of Principa Components
Fca	Factorial Correspondence Analysi
Bic	Bayesian Information Criterion
R	Spearman Correlation Coefficient
Ibd	Isolation By Distance
Geographical Regions	
Ihr	Indian Himalayan Region

Eh	Eastern Himalaya
Wh	Western Himalaya
Chemicals & Reagents	
Edta	Ethylenediaminetetraacetic Acid
Nacl	Sodium Chloride
Guhcl	Guanidine Hydrochloride
$Mgcl_2$	Magnesium Chloride
Ph	Potential Of Hydrogen
Mm	Millimolar
Units Of Measurement	
Kg	Kilogram
G	Gram
M	Meter
Cm	Centimeter
Km^2	Square Kilometre
Ml	Millilitre
µl	Microlitre
°C	Degrees Celsius
Mya	Million Years Ago
Bp	Before Present
Computing And Referencing	

Fig.	Figure

ABSTRACT

The Himalayan blue sheep (*Pseudois nayaur*), commonly known as bharal, is a high-altitude caprine species endemic to the rugged terrains of the high Himalayas. Although classified as a species of "Least Concern" by the International Union for Conservation of Nature (IUCN), significant ambiguities persist regarding its taxonomic delineation and population structure. These uncertainties are further compounded by the paucity of comprehensive genetic data, particularly concerning populations within the Indian Himalayan Region (IHR), where geographical isolation and environmental heterogeneity may have promoted evolutionary divergence. The present study aims to elucidate the phylogenetic relationships, genetic diversity, and population structure of *Pseudois nayaur* across its Indian range, with a specific focus on populations inhabiting the eastern and western Himalayas. Employing a molecular phylogenetic approach, this investigation integrates mitochondrial DNA (mtDNA) markers—principally the cytochrome *b* gene, control region and the complete mitogenome—with nuclear microsatellite markers to assess lineage differentiation, historical demography, and gene flow. Non-invasive and ethically compliant sampling strategies were utilized to obtain biological material, ensuring minimal disturbance to wildlife. Laboratory analyses, including DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing, were followed by bioinformatic assessments using tools such as DnaSP, Arlequin, STRUCTURE, BEAST, and PopART. Phylogenetic reconstructions revealed distinct mitochondrial haplotypes between eastern and western Himalayan populations, suggesting limited gene flow and historical isolation likely driven by topographic and climatic barriers. The genetic differentiation observed was consistent with the theory of allopatric divergence facilitated by the orogenic and glacial history of the Himalayas.

Furthermore, the study confirms the phylogenetic placement of *Pseudois nayaur* within the subfamily Caprinae, identifying close evolutionary affinities with *Ovis* and *Hemitragus* species while supporting its unique lineage status. These findings have important implications for conservation biology, as they suggest the existence of evolutionarily significant units (ESUs) that merit region-specific management interventions. The bharal's role as a herbivore and primary prey for apex predators such as the snow leopard (*Panthera uncia*) further underscores its ecological significance in alpine ecosystems.

In conclusion, this research contributes to the resolution of long-standing taxonomic am
and highlights the necessity of incorporating molecular data into conservation pla
advocates for the implementation of landscape-level conservation strategies aimed at e
genetic connectivity and mitigating the impacts of habitat fragmentation. By provid
insights into the evolutionary history and genetic architecture of *Pseudois nayaur*, t
advances the broader objectives of high-altitude biodiversity conservation in th
Himalayas.

Chapter-1

Introduction

1.1 Background

The ‘Bharal’ (*Pseudois nayaur*) or ‘blue sheep’ is classified as a 'Least Concern' species by the International Union for Conservation of Nature (IUCN). According to the Indian Wild Life (Protection) Act, 1972, it is protected under Schedule I and also listed in Appendix III of the Convention on International Trade in Endangered Species of Flora and Fauna (Harris, 2014). The species is probably found in Tajikistan (Wilson and Reeder, 2005) while it is very common in India, Nepal, Bhutan, China, and northern Pakistan (Coogan et al., 2014). Bharal are predominantly common in the southern slopes from Ladakh to Arunachal Pradesh (Schaller 1977, Mallon 1991; Fox et al., 1991, Sathyakumar 2001, Mishra et al., 2004, Suryavanshi 2010) in the Indian Himalayan Region (IHR) (fig. 1.1). One of the most commonly encountered wild ungulate the blue sheep is the major prey for snow leopard and wolf. It is primarily a grazer, although it could consume herbs and bushes during times when there is a grass shortage (Awasthi et al.,2003). It is a favored prey of snow leopards (*Panther uncia*) and Himalayan wolves (*Canis lupus*); however, juveniles are reported to be predated upon by red foxes (*Vulpes vulpes*) too (Sharma and Lachungpa, 2002).

In the Himalayan region, the elevation range of Bharal habitat is between 2,500 and 5,400 m (Schaller 1977, Aryal et al., 2016). Two species of the genus *Pseudois* are classified based on body size: the Bharal (*P. nayaur*) and the dwarf Bharal (*P. schaeferi*). *Pseudois nayaur* is further divided into two subspecies: *P. n. nayaur*, which predominantly inhabits the western Himalayas, and *P. n. szechuanensis*, reported from the eastern Himalayan region (Joshi et al., 2022). However, the taxonomic status of *P. n. szechuanensis* remains uncertain, as it has not been formally recognized by the IUCN due to insufficient supporting data. The easternmost population of *P. n. szechuanensis* occurs in the Helan Mountains and differs markedly from Bharal in China's Qinghai, Gansu, and Sichuan provinces. The distribution of the dwarf Bharal (*P. schaeferi*) in India remains unclear (Wang et al., 2000; Shackleton, 1997).

Although several studies have examined its current status, distribution, and phylogenetics, the genetic structure and population boundaries of the Bharal remain poorly understood. Previous research has focused on the mtDNA marker Cytochrome *b* (Cyt *b*), which identified two lineages of Bharal in the Indian Himalayan highlands (Joshi et al., 2022). Furthermore, populations in the Himalayan mountains and the Tibetan Plateau are geographically distinct. DNA analysis has confirmed that Bharal in the Tibetan mountains belong to *P. nayaur* and specifically to the subspecies *P. n. szechuanensis*. Based on nuclear genes, the Helan population was also classified as *P. n. szechuanensis*. However, no comprehensive data exist on the status or distribution of these Bharal clades in India. Consequently, the taxonomic classification of Bharal populations across different geographic regions remains unresolved. For Bharal in India in particular, complete mitogenome sequencing may be especially valuable for clarifying the genetic status of multiple populations.

Fig. 1.1 The Bharal: major prey for the Snow Leopard

1.2 Taxonomic Status of Bharal (*Pseudois nayaur*)

The Bharal, commonly referred to as *Pseudois nayaur* and frequently referred to as blue sheep, is a fascinating wild ungulate that has evolved to thrive in the High altitudes Himalaya (>3,000m). Based on its evolutionary history and ecological adaptations, the species is taxonomically positioned within the Animal Kingdom (Wilson & Reeder, 2005).

Pseudois nayaur is classified as a multicellular, eukaryotic organism, categorizing it under the Kingdom *Animalia*. It belongs to the Phylum *Chordata* and Subphylum

Vertebrata respectively, which respectively comprise all creatures with a notochord and a spinal column. Class Mammalia includes this warm-blooded vertebrate with mammary glands and body hair (Nowak, 1999). It is part of the Subclass Theria, which consists of live-bearing species, and the Infraclass *Placentalia*, which comprises all placental mammals, within mammals. It is then further classified as part of the varied clade of mammals known as Superorder *Laurasiatheria*, which include carnivores and ungulates (Hassanin et al., 2012).

The Bharal belongs to the Order *Artiodactyla*—even-toed ungulates—more especially, the Suborder *Ruminantia*, which comprises herbivores with a sophisticated, multi-chamber stomach suitable for breaking down fibrous plant material. More specifically, it belongs to the Subfamily *Antilopinae*, which consists of agile, grazing ungulates; it is attributed to the Family Bovidae, renowned for antelopes, goats, and sheep (Groves & Grubb, 2011). Bharal belongs to the Tribe *Caprini*, are evolutionary relatives of genuine sheep and goats. *Pseudois nayaur* is the only inhabitant of the monotypic genus *Pseudois*. This categorization not only represents its biological characteristics, but also emphasizes its ecological relevance in Indian Himalayan alpine environments.

1.3 Distribution Range of *Pseudois nayaur*

The Bharal is distributed in the Himalayan region that forms the norther border for India, Nepal, Bhutan, Myanmar and Pakistan. In Tibetan region of China (Gansu, South Inner Mongolia (Mittermeier 2011), 'the Ningxia-Inner Mongolia border, Qinghai, Shaanxi, Sichuan, Tibet, south-eastern Xinjiang Uyghur Autonomous Region, and northern Yunnan), and most likely Tajikistan (Shackleton 1997).

- **China:** Its range extends from western Tibet to southwestern Xinjiang (Schaller et al. 1977), where they exist as small populations in the mountains surrounding the western margin of Aru Co, and then eastwards with scattered populations across the autonomous territory. It may also be found in the Kunlun Mountains, and southern Xinjiang. In addition to the Qilian and related Gansu Mountain ranges, it can be found in the majority of the mountain ranges in western and southern Qinghai, as well as in eastern Sichuan and northwest Yunnan. The Helan Shan, which constitute the western boundary of the Ningxia Hui Autonomous Region (with Inner

Mongolia), appear to represent the easternmost extent of its current distribution (Harris 2014).

- **Bhutan:** It might be found at 4,000 to 4,500 meters above sea level in northern Bhutan (Baillie 2004, White 1909).
- **India:** Although the extent of the Bharal's eastern distribution along the northern boundary of Arunachal Pradesh is still unknown, the species is fairly constantly dispersed across the northern Himalayan and Trans-Himalayan areas of India (Rawal et al., 2013). They are very widespread in portions of Spiti and the upper Parbati valley in northern Himachal Pradesh, as well as in numerous regions of eastern Ladakh (Jammu and Kashmir). Bharal might be found in eastern Arunachal Pradesh, on the slopes of the Khangchendzonga massif in Sikkim, near Badrinath in Uttar Pradesh, and in the Govind Pashu Vihar Wildlife Sanctuary and Nanda Devi National Park. The existence of Bharals has only recently been verified in the northwest region of Arunachal Pradesh, near to its borders with China and Bhutan (Harris 2014).
- **Nepal:** It is roughly distributed to the north of the Greater Himalaya, from the border with India and Tibet in the extreme northwest, eastward via Dolpo and Mustang to Gorkha district in north-central Nepal. After that, it reappears in Nepal in at least two remote locations: Lamobogar and the southwest slopes of Kanchenjunga near to the Indian border with Sikkim (Schaller 1977, Heinen 1992). These two most likely have connections to more extensive communities in Tibet on the other side of the border (Aryal et al, 2013).
- **Pakistan:** The upper Gujerab and upper Shimshal valleys, as well as the region east of Shimshal pass (District Gilgit), which contains a portion of Khunjerab National Park, comprise its primary distribution range in Pakistan (Schaller 1977; Roberts 1977; Ghalib et al., 2019). One animal from Khunjerab Pass has been observed recently outside of these locations. Previously, Roberts (1977) suggested that, its occurrence (with proof) near Shigar and the Baltoro glacier (District Baltistan), however, there is no genuine information from this location.

1.4 IUCN Status of *Pseudois nayaur*

The Bharal is categorized as "Least Concern" in IUCN Red List due to its widespread distribution, estimated high population, and the likelihood that its population decline will be quite lower than the rate required to be listed as threatened (fig. 1.2). There are no population estimates available for the entire range. However, if the available numbers are added together, the total population could be as high as 414.000 (likely

an excess) or as low as 47.000 (likely a very cautious estimate). Nearly all Bharals live in robust, mountainous areas. It is most likely lacking from large regions (like basins) without severe terrain. It appears to be found in almost all suitable mountainous terrain, from the Himalaya in the south to the Qilian Mountains in the north (Gansu and Qinghai provinces), therefore this does not necessarily imply that populations are divided. In western China, the Greater Bharal is most likely the most common ungulate (Buzzard et al., 2018; Feng et al., 2024).

- **China:** The majority of population size extrapolations in China are not well-founded and should be treated with care. In contrast to other species, field examinations of subjective and informal descriptions of *P. nayaur* consistently affirm the species' presence and general abundance. As a result, even though specific numbers in Chinese literature could be off, all available data indicates *P. nayaur* becoming ubiquitous and comparatively abundant in suitable habitats in Chinese regions of the Tibetan plateau. *P. nayaur* was described as "relatively abundant" in Western China (Shackleton 1997), with a total population of over 10,000 and a variety of (uncertain) local density estimates. The most of these 10.000 were likely overestimates that were extrapolated for the Arjin Shan Nature Reserve. The number of 10.000 in the western part of the Chinese range is not necessarily out of the question, though, as several locations within the present range were not included in this estimate (Schaller 1997 thought that at least 10.000 inhabited the Chang Tang Nature Reserve in western Tibet). Wang (1998) claimed a Chinese extrapolation of more than 1.200.000 in Qinghai province (of which 267.000 were in "eastern" Qinghai), which is most likely a significant exaggeration. Zhao et al., (2021) estimated between 5,000 and 9.000 Bharal in the Helan Shan (Inner Mongolia), however their extrapolations were based on transects that were not set at random (Harris 2014). Even greater estimates are provided by some estimations for this region: 10,000
- **India**: Shackleton (1997) references a Fox et al. (1991) estimate of "a minimum of 11.000" in Ladakh, India.
- **Nepal:** Schaller (1998) presented an illustration indicating 1.947 to 2.561 animals in regions included within Nepal, despite Shackleton (1997) reporting a "conservative estimate of 10,000 animals." Pakistan: According to published, the species is not as scarce as originally believed, according to Shackleton. In 1992, the latest estimates put the number of animals at 2,000 and 2,500.

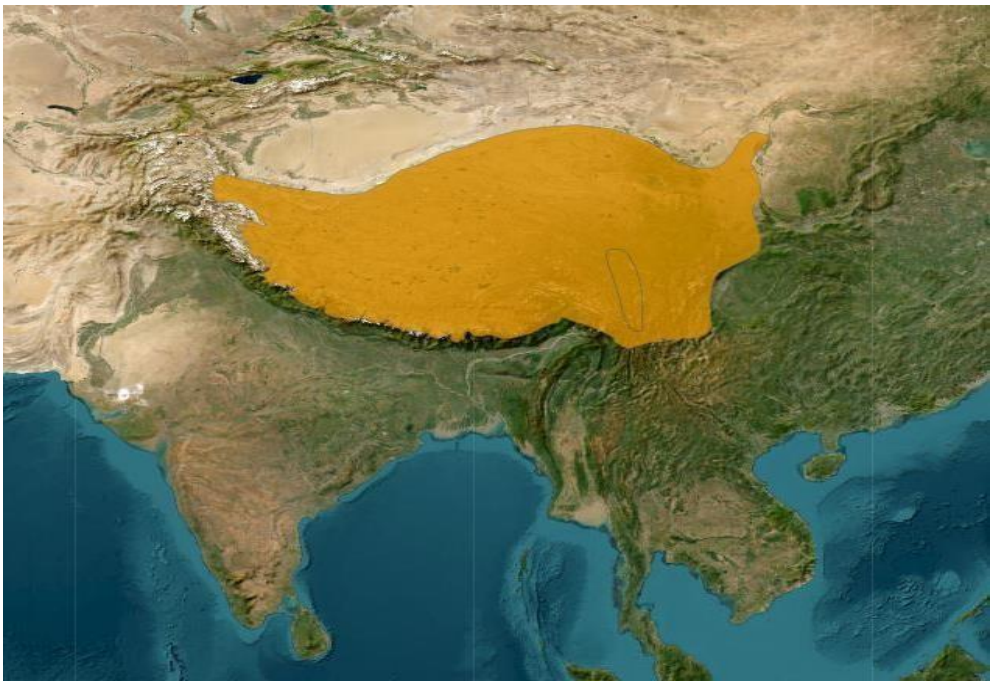

Fig. 1.2 Distribution of the Bharal (*Pseudois nayaur*) Source: IUCN Red List of Threatened Species

1.5 General appearance and Morphology

The bharal, or Bharal, is a robust ungulate with muscular limbs and a broad chest, well adapted for life in steep, rocky terrain (Schaller 1997, 2000). Its relatively short legs minimize heat loss and lower the centre of gravity. Morphologically, it displays traits of both sheep and goats. *P. nayaur* lacks most sheep-typical glands—including the inguinal, preorbital, and interdigital glands—and does not possess a beard, strong odour, or knee callus (traits common in goats). Goat-like features include a wide, flat tail with a bare underside, large dewclaws, distinct limb markings, and certain cranial characteristics (Wilson & Mittermeier, 2011).

Adult males measure 120–140 cm in body length and 80–91.4 cm at the shoulder, weighing 60–75 kg. Females weigh 35–55 kg (Schaller, 1997, 2000). Male horns, ranging from 38–76 cm (occasionally >82 cm), sweep outward, then downward and backward, with slightly curled tips; female horns are shorter (10–20 cm). Horns grow gradually with age and display annual growth rings (Shackleton, 1997). The tail measures 10–20 cm, is broad and flat, and has a naked underside. The species usually has 2n = 54 chromosomes, although one record from China reported 56 (Schaller,

1997, 2000). While bharal may live up to 15 years in captivity, individuals older than 10 years are rarely encountered in the wild.

- **Coloration and Pelage**

  Coat colour changes seasonally, from bluish-grey in winter (origin of the name "Blue sheep") to brownish-grey in summer (Shackleton, 1997). Mature males have a distinctive black chest and neck, absent in females. The underparts are white, occasionally golden-brown, with a conspicuous black flank stripe, more pronounced in older males. Forelegs are black in front and white behind; the rump patch is white and the face dark brown. The tail is predominantly black with a bare underside.

- **Habitat and distribution**

  P. nayaur inhabits diverse high-altitude habitats, typically between 2,500 and 5,500 m a.s.l. (Shackleton, 1997). It generally avoids forests, favouring open grassland slopes near cliffs for predator evasion. Habitat preferences vary geographically—alpine sedge meadows in Xizang, montane grasslands and shrublands in the Helan Mountains of Inner Mongolia, and mixed grass-bare ground mosaics in Nepal (Fox et al., 1991). It thrives even in marginal environments and frequently overlaps with domestic livestock at high elevations (Harris, 2014; Mishra et al., 2004; Surywanshi et al., 2010).

- **Feeding Ecology**

  The diet is dominated by graminoids in summer (up to 80%) and sedges in other seasons, with forbs abundant in spring and shrubs increasingly important in autumn and winter. In northern China, graminoids form 36.7% of the winter diet and 58.8% in summer, with browse reaching up to 50% (Harris, 2014). In Nepal, graminoids also dominate, followed by a balance of forbs and browse (Fox et al., 1991). Commonly eaten plants include *Arundinella*, *Danthonia*, *Festuca*, *Trisetum*, *Poa*, *Berberis*, and *Juniperus* (Miller 1987).

- **Interspecific Conflict**

  Bharal often share habitats with other herbivores, such as Siberian ibex, Tibetan argali (in Ladakh), and domestic livestock. Overlap with livestock (goat and sheep) is common, particularly in summer, can lead to competition for graminoids (Mishra et al., 2004; Bhatnagar et al., 2006).

- **Predation and mortality**

  Mortality peaks in late winter and spring due to climatic stress and food scarcity (Schaller, 2000). Predation is significant where large carnivores are present, especially snow leopards, which consume 11–24% of the local bharal population annually in some areas (Fox et al., 1991). Other predators include wolves, leopards, dholes, red foxes, and raptors such as steppe eagles.

- **Development and Reproduction**

  Bharals achieve sexual maturity about 1.5 years old, but males seldom reproduce successfully until age 7. The mating season lasts from December to January, with most births taking place between May and June (Schaller, 1977, 2000). Courtship consists of elaborate demonstrations; dominant males older than five years usually control breeding. With a 25day cycle, an estrus lasts 1 to 4 days. Usually delivering a single lamb, ewes have a 160-day gestation period. At six months, weaning takes place; temporary lambs' nurseries (creches) of a few mothers have been seen (Shackleton, 1997).

- **Social Structure and Behavior**

  Bharals are diurnal and most active in the early morning and late afternoon. During the rut, midday activity drops, but afternoon activity increases later in the season. Socially, Bharals are sociable and create herds ranging in count up to three hundred thousand. On the Tibetan Plateau, female herds average 14.5 members; mixed herds approximately 23.2. Limited resources in winter causes group size to often decline (Schaller, 2000). The Helan Mountains have seen lower average herd counts. Males blend into mixed herds during rutting season; up to 80% of them join during peak breeding time. In ideal environment, population density falls between 3.6 and 5.3 individuals/km². Males have a home range of 3-7 km², whereas females have a range of 2.5-5 km² during winter (Harris, 2014).

- **Demographic Trends**

  The sex and age ratios change annually, with around 93.7 males per 100 females in spring, 126.9 in fall, and 91.7 in winter (Fox et al., 1991). Of the population, 34% are adult males, 32% are ewes, 15% are yearlings, and 19% are lamb. Lamb-to--ewe ratios vary; they peak in autumn (70%) then fall in March (52.5%). On the Tibetan

Plateau, yearling to female ratios usually run from 26 to 40 per 100 (Schaller, 2000; Mishra et al., 2004; Suryawanshi et al., 2010).

1.6 Ecological Significance in Alpine Ecosystems

The Bharal (*Pseudois nayaur*) exerts a significant ecological influence on alpine habitats, particularly in the Himalayas and the Tibetan Plateau. As a keystone herbivore, it plays a pivotal role in shaping predator–prey dynamics and influencing the structure and composition of plant communities (Schaller, 1998; Mishra et al., 2004). One of its most critical ecological functions is serving as the principal prey species of the snow leopard (*Panthera uncia*). In regions such as Manang and Mustang in Nepal, Bharal constitute 40–60% of the snow leopard's diet (Fox et al., 1991). This strong trophic association means that snow leopard abundance and distribution are often closely correlated with the presence and population density of Bharal (Mishra, 2004).

Bharal substantially alter grazing pressure on alpine meadows and shrublands, thereby affecting plant biomass and community composition. When population density exceeds 5 individuals per km², vegetation tends to exhibit reduced grass height, increased areas of bare ground, and a prevalence of grazing-tolerant plant species (Bhatnagar et al., 2006; Mishra et al., 2004). Their seasonal dietary shifts—primarily grazing on graminoids during summer and switching to browse during winter—help maintain vegetation heterogeneity throughout the year and contribute to nutrient cycling in alpine systems (Fox et al., 1991; Lü et al., 2000).

Furthermore, Bharal contribute to nutrient redistribution across alpine pastures through the deposition of urine and feces. Their extensive home ranges, typically between 2.5 and 7 km², facilitate the widespread dispersal of organic matter, enhancing soil fertility and stimulating alpine grassland productivity (Wilson & Mittermeier, 2004; Harris, 2014). Areas with high dung deposition often function as localized nutrient hotspots, benefiting both wild herbivores and domestic livestock in shared grazing landscapes, such as those inhabited by yaks and goats (Namgail et al., 2004). However, cohabitation with cattle can lead to interspecific competition, particularly in summer when dietary overlap is greatest. In such cases, overgrazing by cattle can degrade habitats and displace Bharal to suboptimal feeding areas (Bhatnagar et al., 2006; Mishra, 2004). In contrast, under balanced grazing regimes,

Bharal act as natural regulators of vegetation succession, preventing excessive shrub encroachment and thereby maintaining the ecological equilibrium of alpine meadows (Schaller, 1997).

Finally, the Bharal functions as an indicator species for assessing the overall health of alpine ecosystems. Given its sensitivity to habitat degradation, interspecific competition, and predation pressure, fluctuations in its population size and distribution can serve as early-warning signals of ecological disturbance (Shrestha & Wegge, 2008; Mishra et al., 2004). Consequently, its presence, abundance, and behavioural patterns are essential parameters for monitoring and conserving biodiversity in high-altitude environments.

1.7 Significance of Studying Bharal in the Indian Himalayas

The Bharal (*Pseudois nayaur*) holds substantial ecological, environmental, and socio-cultural importance in the Indian Himalayan region. Comprehensive understanding of its biology, distribution, and genetic diversity is vital for advancing conservation research and implementing sustainable ecosystem management strategies (Schaller, 1977).

- **Ecological role as a prey species** – The Bharal plays a dual ecological role: as a keystone species and as a principal prey for large predators such as the snow leopard (*Panthera uncia*), a globally threatened felid. Its abundance directly influences predator population sizes, making it essential for maintaining ecological balance (Valentová et al., 2022). Through selective grazing, Bharal also shape the structure and composition of alpine vegetation, thereby influencing the availability of resources for other herbivores and affecting the entire trophic network of the trans-Himalayan ecosystem (Rawat, 2015).
- **Indicator of ecosystem health** – Owing to their sensitivity to habitat alterations, Bharal serve as reliable bio-indicators in fragile alpine environments. Fluctuations in their population dynamics, movement patterns, or physical condition can signal broader ecological changes, including climate variability, habitat fragmentation, or anthropogenic disturbances (Namgail et al., 2009). Continuous monitoring of Bharal populations thus provides early-warning signals of environmental degradation and helps in formulating targeted conservation interventions.

- **Relevance to biodiversity and conservation goals** – Research on Bharal is directly linked to national and global biodiversity conservation objectives, particularly in the Himalayan biodiversity hotspots (Rodgers & Panwar, 1988). As a high-altitude-adapted species, Bharal contribute to the region's unique ecological diversity. Conserving their populations also safeguards the integrity of the alpine ecosystem and its associated native flora and fauna. Furthermore, molecular studies of their genetic diversity enhance understanding of their adaptive potential and resilience—knowledge that is increasingly important in the context of climate change and intensifying human–wildlife conflicts (Karmacharya et al., 2022; Choudhury et al., 2018).
- **Cultural and socio-economic value** – For numerous Himalayan indigenous communities, the Bharal has deep cultural and socio-economic significance. It features prominently in local mythology, spiritual beliefs, and traditional subsistence hunting practices (Beall & Goldstein 1992). In recent decades, its presence has also driven eco-tourism, particularly wildlife tourism centered on snow leopard tracking, which generates income and employment in remote high-altitude villages (Dutta et al., 2024). As such, conserving Bharal is critical to community-based conservation models, ensuring that ecological protection is aligned with the socio-economic development of local populations.

1.8 Molecular Phylogeny: Concepts and Importance

Phylogenetics is the scientific discipline concerned with elucidating evolutionary relationships among biological entities species, individuals, or genes collectively referred to as taxa. By comparing genetic, morphological, or molecular data, phylogenetics enables researchers to reconstruct divergence patterns and identify common ancestry (Nei & Kumar, 2000; Felsenstein, 2004).

Molecular phylogenetics, a specialised subfield, focuses on evolutionary relationships inferred from molecular sequences, primarily DNA, RNA, or proteins (Yang & Rannala, 2012). This approach has revolutionised our understanding of the evolutionary history of life by enabling the construction of phylogenetic trees that visually depict descent and divergence patterns (Hillis et al., 1987). Such analyses not only illuminate the molecular mechanisms underpinning evolution but also support predictions of potential future genetic variation (Page & Holmes, 2009)

The importance of phylogenetics lies in its ability to provide a conceptual framework for examining the evolutionary dynamics of genes, genomes, and species (Maddison & Schulz, 2001). It offers insights into the present organisation and function of genetic material while informing our understanding of its evolutionary trajectory. Consequently, molecular phylogenetics has become integral to a range of scientific disciplines, including systematics, ecology, medicine, and biotechnology (Lemey et al., 2009).

1.8.1 Applications of Molecular Phylogenetics

- **Taxonomy and classification** – Molecular phylogenetic analyses based on sequence data have significantly advanced our understanding of evolutionary relationships among species, enabling more accurate and robust taxonomic classifications (Tautz et al., 2011; Hibbett et al., 2007). By integrating molecular evidence with traditional morphological assessments, these studies have greatly refined the conventional Linnaean system, particularly in the classification of newly discovered or cryptic species.
- **Forensic science** – Phylogenetic methodologies are increasingly employed in forensic investigations through the analysis of DNA evidence. When applied to criminal cases, paternity disputes, and food authentication or safety assessments, these approaches provide scientifically rigorous evidence that is admissible in legal proceedings (Salemi & Vandamme, 2003).
- **Pathogen tracking and public health** – Molecular phylogenetics is indispensable in understanding the origin, evolution, and transmission dynamics of infectious diseases. Sequence data can be used to infer relatedness among emerging pathogens, reconstruct their evolutionary histories, and identify transmission pathways (Drummond et al., 2005; Pybus & Rambaut, 2009). Such insights are critical for informing epidemic control strategies, guiding vaccination campaigns, and shaping public health policy (Grenfell et al., 2004).
- **Conservation biology** – Phylogenetic data play a vital role in conservation planning by helping to priorities species and lineages for protection. Beyond safeguarding individual species, conservation strategies informed by phylogenetics aim to preserve evolutionary diversity, a key factor in maintaining long-term ecosystem resilience and adaptive capacity (Faith, 1992).

- **Bioinformatics and computational biology** – The development of phylogenetic algorithms has driven major advances in computational biology and bioinformatics. These algorithms underpin many modern software tools used in genomics, transcriptomics, and systems biology, and have applications extending beyond biological research, including linguistics, anthropology, and data science (Swofford, 1993; Tamura et al., 2013).
- **Definition and Role in Evolutionary Biology**

  Molecular phylogeny is the study of molecular sequences to define connections between species or individuals (Nei & Kumar, 2000). Molecular phylogeny offers a strong framework for rebuilding evolutionary trees by means of genetic markers such mitochondrial DNA (mtDNA), Cyt *b,* D-loop (Avise, 2012). These trees allow the discovery of shared ancestral features, common ancestors, and divergent evolutionary routes as well as the genealogical links across species (Hillis et al., 1987). Molecular phylogeny is important in evolutionary biology because it can precisely monitor the evolutionary history of living forms, supporting ideas regarding evolutionary processes, adaptability, and the genetic foundation of species diversification (Yang & Rannala, 2012).

  Molecular phylogeny can also help to demonstrate complicated evolutionary processes like hybridization, gene duplication, and horizontal gene transfer, which are difficult to infer from traditional morphological traits alone (Doolittle, 1999; Mallet, 2005). Scientists could produce predictions about species' evolutionary behaviors using DNA sequence analysis, giving essential data for evolutionary models (Page & Holmes, 2009).

- **Application in species identification and taxonomy**

  Molecular phylogenetics has become critical in species identification and taxonomy, especially when traditional morphological categorization is difficult or ambiguous (Tautz et al., 2011). This technique allows for more precise descriptions of evolutionary connections by detecting genetic differences and similarities that are not obvious at the phenotypic level. Molecular markers such as mitochondrial DNA, microsatellites, and single nucleotide polymorphisms (SNPs) are commonly used to distinguish closely related species or populations that appear physically similar but differ genetically (Hebert et al., 2003; Avise, 2000).

Furthermore, molecular phylogenetics has enhanced the Linnaean categorization system by giving a more exact approach for classifying species and genera based on evolutionary lineage rather than observable features (Wiens & Penkrot, 2002). Using molecular data in taxonomy has resulted in the identification of cryptic species—species that are genetically unique but physically identical—as well as reclassification of several species (Bickford et al., 2007).

- **Bharal's Phylogenetic Position Within the Bovidae**

  The Bharal (*Pseudois nayaur*) belongs to the Bovidae family of herbivorous animals that includes goats, sheep, and cattle. Bharal are classified by molecular phylogenetic studies as members of the subfamily *Caprinae*, which include genuine sheep, goats, and other closely related animals. Studies using mitochondrial DNA and nuclear genetic markers have shown that the Bharal has a common ancestor with species such as the himalayan tahr (*Hemitragus jemlahicus*) and wild sheep (*Ovis* species), though it diverged from these species around 5-6 million years ago (Fletcher 2020).

  Understanding Bharal's phylogenetic position within Bovidae is critical for investigating evolutionary adaptations to high-altitude settings in the Indian Himalayas. Phylogenetic data sheds light on how Bharal have evolved to the harsh cold, rocky terrain, and scant flora of their environment, as well as how they compare to other mountain-dwelling bovids (Ge et al., 2013).

- **Comparative Phylogenetic Studies in *Caprinae***

  Caprinae, the subfamily comprising domestic and wild sheep, goats, and various relatives, is a varied group having seen major evolutionary changes over millions of years. Comparative molecular phylogenetic studies in *Caprinae* enable scientists to follow the evolutionary links and diversification of these species, so providing vital new perspectives on their adaptive features including resistance to diseases, high-altitude survival mechanisms, and dietary specialization (Groves & Shields, 1996).

  Phylogenetic investigations employing DNA sequencing have shown a complicated evolutionary history within Caprinae, with several speciation events associated with geographic isolation, climate changes, and ecological adaptations (Manceau et al., 1999). These findings demonstrate the divergence of species such as Bharal, wild goats, and domestic sheep from a common ancestor and shed information on the genetic basis of features like as coat color, body size, and behavior. Comparative

studies in *Caprinae* also help to explain the evolutionary relevance of hybridization events between closely related species, which are typical in this group.

1.9 Evolution and Divergence of Bharal

- **Ancient Biogeography of the Himalayas of India**

The Indian Himalayas are a distinct and complicated geographical location that has been critical to the evolutionary history of numerous species, including the Bharal (*Pseudois nayaur*) (Mani, 1974; Rawat, 2005). The Himalayas have been a center of speciation as well as a physical barrier. The region's past biogeography has been impacted by geological upheavals, climate alterations, and vegetation changes, all of which have had an impact on species distribution and divergence. During the Cenozoic age, the Himalayan Mountain range was uplifted, resulting in isolated high-altitude habitats that favored the development of species suited to severe conditions (Zhisheng et al., 2001).

The Indian Himalayas have supplied Bharal with distinct ecological niches. Over millions of years, geographical isolation caused by difficult mountain topography has fostered species and population divergence, leading to the genetic uniqueness of Bharal in this region. The geographical variety of the landscape *viz.* valleys, cliffs, and ridges have greatly influenced population patterns and gene flow among Bharal communities.

- **Divergence Timelines using Molecular Clock Models**

Molecular clock models are commonly employed to predict species divergence timeframes using genetic mutation rates across time (Ho & Larson, 2006). By examining genetic variations between species or groups, scientists can potentially identify how common ancestors lived and at what point specific lineages parted ways. Molecular clock studies of Bharal indicate separation from other closely related species in the *Caprinae* subfamily (e.g., wild sheep and goats) occurred roughly 5–6 million years ago. This chronology aligns with major geological and climatic processes that affected the diversification of high-altitude species in the area: the elevation of the Tibetan Plateau and the strengthening of the Asian monsoon (Molnar et al., 1993).

Molecular clocks are based on the premise that mutations in genetic sequences occur at a reasonably consistent pace across time. The study of nuclear and mitochondrial DNA (mtDNA) has provided light on how environmental events, including the Pleistocene glaciations, could have molded the evolutionary path of the Bharal.

- **Effect of topography and glacial cycles on speciation**

High-altitude animals like the Bharal (Hewitt, 2000) evolved profoundly during the repeated cycles of glacial expansion and retreat of the Pleistocene glaciations. These climatic changes induced alterations in flora and habitat availability, which most likely resulted in population fragmentation and genetic isolation. As glaciers receded, Bharal populations were left in isolated refugia, where they adapted to unique climatic circumstances. This seclusion helped communities all throughout the Himalayas to undergo genetic divergence and speciation. Furthermore, restricting gene flow between populations is the geographical complexity of the Himalayan area, with its high slopes, valleys, and ridges. These physical constraints enabled the evolution of unique genetic fingerprints in many groups and encouraged genetic difference (Nybom et al., 2014).

- **Population Genetics of Bharal**

- **Principles of Population Genetics:** Population genetics is the study of genetic diversity within populations and the mechanisms throughout time that influence this variation including mutation, gene flow, genetic drift, and natural selection (Garant, 2007). Population genetics clarifies how genetic diversity is dispersed among several populations in the Indian Himalayas, which may experience varied degrees of gene flow and environmental stressors in Bharal.

- **Importance of Genetic Diversity and Gene Flow**

The adaptability and long-term survival of a species are contingent upon genetic diversity. High genetic variety makes populations more likely to adapt to environmental changes, fend off illnesses, and preserve ecological stability (Erard et al., 2004). Maintaining genetic variety is particularly crucial for Bharal in response to habitat loss, climate change, and other human challenges (Biswas et al., 2025).

Genetic diversity is preserved and inbreeding risk decreases through gene flow—that is, the movement of genetic material across populations (Slatkin, 1987). However, in

Bharal, topographical factors such as high slopes and deep valleys impede gene flow, promoting genetic isolation across populations.

- **Genetic Variation and Population Structure**

  Population structure in genetics is the arrangement of genetic variation between groups across space. Geographic isolation, climate events, and habitat uniqueness influence genetic structure in Bharal. Molecular marker studies employing mtDNA and microsatellites have showed considerable genetic difference between populations in the Western and Eastern Himalayas, consistent with isolation by distance and geographic barriers (Qiu et al., 2015; Wei, 2016). Genetic heterogeneity across populations helps researchers in understanding evolutionary forces and environmental limitations that determine local adaption and possible speciation processes (Avise, 2000).

- **Factors Affecting Genetic Variation (e.g., isolation, fragmentation)**

  Geographic isolation and habitat fragmentation are among the several elements influencing the genetic variety of Bharal. Habitat fragmentation, which is frequently caused by manmade pressures like as roads, dams, and grazing, breaks habitat continuity and limits feasible population sizes (Fahrig, 2003). Smaller, isolated populations are more likely to undergo loss of genetic diversity, inbreeding, and genetic drift (Frankham, 1996). Further contributing to demographic difference is natural isolation resulting from the difficult Himalayan topography, which limits dispersion and gene flow (Cun 2010). These isolated populations may have limited ability to adjust to fast environmental changes, making them more susceptible to extinction.

1.10 Genetic Markers Used in Bharal Studies

- **Mitochondrial DNA (mtDNA): Cytochrome b & Control Region**

  Mitochondrial DNA (mtDNA) is widely used in evolutionary and phylogenetic investigations owing to its maternal inheritance, absence of recombination, and relatively high mutation rate (Avise et al., 1987; Ballard & Whitlock, 2004). For evaluating genetic diversity and inferring evolutionary connections in Bharal (*Pseudois nayaur*), mtDNA markers including the cytochrome *b* gene and the control region (D-loop) have shown to be extremely valuable. The cytochrome *b* gene, a component of the mitochondrial respiratory chain complex, offers critical information on genetic divergence across and within species (Irwin et al., 1991). On

the other hand, the relatively highly variable control region is rather helpful for understanding maternal lineage variation and spotting sub species-level changes (Brown et al., 1986).

- **Microsatellites and Their Applications in Nuclear DNA**

  Microsatellites, or simple sequence repeats (SSRs), are tiny DNA sequences that occur in tandem throughout the nuclear genome. These markers are highly polymorphic and codominant, making them appropriate for investigations into population structure, gene flow, genetic drift, and inbreeding (Jarne & Lagoda, 1996; Selkoe & Toonen, 2006). In Bharal investigations, microsatellite markers have been successfully applied to measure genetic variation across geographically distant populations and assess population interaction in face of habitat fragmentation. Their nuclear source suggests they reflect biparental inheritance, therefore providing another perspective on the matrilineal mtDNA data.

- **Benefits of combining microsatellite and mtDNA data**

  Microsatellite and mtDNA data combined offers an exhaustive overview of genetic variation. While mtDNA defines maternal lineage and previous demographic events, microsatellites provide high-resolution perspectives of modern gene flow and population structure (Petit et al., 2005). By exposing both deep differences and surface genetic structure, this combined approach has proved helpful in Bharal research for reconstruction of evolutionary histories and identification of conservation units. In animals such as Bharal, which live in difficult and scattered alpine habitats where historical isolation and current gene flow interact to affect genetic patterns, the complementary character of these markers is particularly important.

1.11 Methodology

- **Methods of sampling for population genetics of Bharal**

  Sampling strategies are critical for population genetics studies on Bharal (*Pseudois nayaur*), since they allow researchers to acquire representative genetic data from a variety of habitats in the Himalayan region. An optimal sampling strategy is required not only for quantifying genetic diversity within populations, but also for understanding genetic differences across populations and detecting the evolutionary

processes that shape their genetic structure. The key components of various sampling procedures are mentioned as follows:

- **The Regional coverage**

  Sampling locations have to be specifically chosen to cover a wide geographic spectrum of Bharal habitats including different mountain ranges, valleys, and biological zones inside the Himalayas. This approach allows researchers to document genetic variation brought about by environmental factors like gradient of temperature, types of vegetation, and height. Scientists might investigate how these environmental variables impact genetic variation and adaptation in Bharal populations by sampling across many settings, providing insight into how these populations have evolved in response to their specific circumstances.

- **Population Representation**

  Accurate genetic studies need enough samples from every population to evenly show genetic factors. The sample sizes must be enough to ensure the population's proper genetic variety and avoid data biases. Efforts at sampling should consider the demographic composition and size of the population. This entails gathering samples from various individuals living in every society contemplating any genetic diversity brought about by aspects including sex, age, and social structure. Collecting different samples from all throughout the population helps researchers to ensure the robustness of the genetic analysis and gain a better knowledge of genetic variation.

- **Non-invasive Sampling Techniques**

  Non-invasive sampling techniques are typically employed provided the difficult terrain and conservation issues related with Bharal. These methods help the animals relax and yet provide valuable genetic material for research. Common non-invasive sampling techniques are faecal samples, hair samples (from places), and tissue samples (from naturally occurring deaths). These techniques assist ethical considerations of the study by offering genetic material suitable for DNA extraction and subsequent genetic analysis without physically harming or stressing the animals.

- **Genetic Connectivity**

  Beneficial sampling techniques should consider the genetic connects between populations. Evaluating the genetic condition of Bharal populations requires an understanding of patterns of gene flow and genetic interaction throughout the terrain.

It examines the impact of landscape features on genetic structure and population survival by identifying natural corridors or barriers to gene flow, such as rivers, valleys, or human-made impediments. Connectivity assessments help in the development of conservation strategies aimed at protecting genetic diversity and enhancing population resilience in the face of environmental changes like as climate change or habitat fragmentation.

- **Monitoring and prolonged studies**

  Long-term monitoring, which includes periodic sampling over several years or generations, is required to follow changes in genetic diversity, population structure, and adaptation across time. Longitudinal studies allow researchers to monitor the effects of environmental disturbances, climate change, and human activities on Bharal populations and their genetic health. Constant data collecting over extended periods of time allows scientists to better understand how these components influence genetic variation and how populations respond to ongoing environmental stresses.

- **Comprehensive and group sampling**

  Collaboration between research institutions, wildlife managers, and local people is essential for the implementation of comprehensive sampling programs. Local experience and knowledge can significantly enhance the success of sample campaigns; also, incorporating locals in conservation programs helps generate more widespread support for the research. Collaboration also promotes data sharing and standardisation of sampling methods across study sites, resulting in relevant genetic data comparisons and meta-analyses. This integrated approach ensures not just scientific rigour but also cultural relevance and fit with local conservation goals.

1.12 Tools & Instruments used for Analysis

- **Sequencher v4.9 and BioEdit were used for sequence editing and alignment**: Raw chromatograms from sequencing reactions were edited for quality and combined into consensus sequences using Sequencher (Gene Codes Corporation, USA). The software detects and corrects ambiguous bases, resulting in high-fidelity sequence output. Following their alignment, the aligned sequences were manually curated using BioEdit, a biological sequence alignment editor (Hall, 1999), which supports editing, alignment, and nucleotide sequence annotations.

- **Nucleotide and haplotype diversity were analysed using DnaSP v5 software:** This was to measure genetic diversity using two parameters:

- Nucleotide diversity (π) is the average fraction of nucleotide variations per site between two sequences randomly selected from the population.
- Haplotype diversity (h) is the probability that two randomly selected haplotypes in a sample are different.

  These indices were calculated using DnaSP v5 (Librado and Rozas, 2009), a powerful program for analysing DNA sequence polymorphism data.

- **Software used for demographic analysis is Arlequin v3.5:** Two main tests are performed:

- **Tajima's D:** An average number of nucleotide difference statistical test for neutrality that contrasts the number of segregating sites. A significant negative Tajima's D indicates to either population increase or clearing selection.
- **Fu's Fs:** A test that detects demographic expansion; significant negative results imply recent population increase or genetic hitch-hiking.

  Arlequin v3.5 (Excoffier and Lischer, 2010) supports several population genetic and demographic studies, hence both tests were carried out using it.

- **BEAST v1.8 (Drummond et al., 2012) was used to estimate phylogenetic relationships and divergence times among haplotypes**

- This Bayesian MCMC-based program can reconstruct time-calibrated phylogenies under different substitution models.
- The posterior distribution of trees and parameters was summarized using Tracer and visualized with FigTree.

- **PopART software was used for the median network joining:** It was to visualize genealogical links between haplotypes.

- The network emphasizes ancestral rather than derived lineages and shows the lowest number of mutational steps between haplotypes.
- The MJN was built using PopART (Population Analysis with Reticulate Trees), which provides an easy-to-use interface for intraspecific data visualizing.

- **MEGA X (Kumar et al., 2018)**: It was used to determine pairwise genetic distances between sequences based on multiple evolutionary models.

- Assessments of phylogenetic and demographic divergence are built on these distances. Population differentiation is measured using pairwise comparisons. GenAlEx v6.5 was used to evaluate genetic differentiation across populations by means of FST values, a standard fixation indicator measuring the fraction of genetic variation ascribed to population subdivision.
- GenAlEx v6.5 (Peakall and Smouse, 2012) an Excel-based population genetics tool was used to compute pairwise FST values.

- **MICROCHECKER v2.2.3**: It was used to test Microsatellite data for possible genotyping mistakes including stuttering, allele dropout, and null allele presence (van Oosterhout et al., 2004). This step ensures the data's integrity and dependability for downstream processing.

- **Microsatellite Diversity Indices measured parameters:** Polymorphic Information Content (PIC), Observed heterozygosity (Ho), Expected heterozygosity (He)

- Basic diversity data were computed using CERVUS v3.0.6, developed by Kalinowski et al., 2007. Understanding population structure depends on knowing the degree of genetic diversity at every microsatellite locus, so these indices represent that at each of them.
- F_{STAT} (Goudet, 2002) was used to compute allelic richness (Ar) and mean inbreeding coefficient (FIS), which account for sample size differences.

- **STRUCTURE v2.3.4 software**: It was used to assign individuals to genetic clusters (K) using a Bayesian technique for population structure inference (Pritchard et al., 2000). It finds admixed people and approximates the percentage of every person's genome belonging to every population.

- **ADEGENET R package:** The ADEGENET package (Jombart, 2008) for R was used to perform Discriminant Analysis of Principal Components (DAPC). This approach is successful at spotting and characterising groups of genetically related people and does not assume Hardy-Weinberg equilibrium.

- **GENETIX v4.05 Software:** Using GENETIX, which permits graphical visualisation of multilocus genetic data to investigate population structure and individual assignment, factorial Correspondence Analysis (FCA) was performed.

- **Alleles in Space v1.0:** Isolation by Distance (IBD) study was done using Alleles in Space to assess the link between genetic distinctiveness and geographic distance (Miller, 2005). This technique for spatial genetic analysis enables one to evaluate if groups separated more apart are more genetically different.

1.13 Laboratory Techniques:

1.13.1 Extraction of DNA

DNA extraction is the process of physically or chemically extracting DNA from biological sources. The primary objective is to separate DNA from other biological elements such proteins, cell membranes, and RNA. In 1869 Friedrich Miescher made first successful DNA isolation. The goal of DNA isolation is to get high-quality, pure DNA devoid of contaminants such as RNA and proteins for further analysis. DNA extraction can be done manually or using commercially available kits, depending on the sample type and desired output.

- **Sources of DNA extraction:** DNA could be extracted from a variety of tissue types, depending on the nature of the research or diagnostic analysis. Blood is a common source as it is easily available and produces outstanding nucleated cells. Other non-invasive or slightly invasive sources of DNA extraction are bodily fluids including saliva, urine, cerebrospinal fluid, and others. In cytogenetic and molecular research especially, fine needle aspiration cytology (FNAC) aspirates cellular material from specific lesions or masses. Moreover, although DNA fragmentation might be a challenge, formalin-fixed paraffin-embedded (FFPE) tissues, which are extensively kept in pathology laboratories, offer valuable archive material for retrospective molecular study. Another excellent source of high-quality DNA suitable for many molecular uses like PCR, sequencing, and gene expression study is frozen tissue sections kept at low temperatures. Basic steps in DNA Extraction:

- **Cell lysis:** Cells disintegrate to release their DNA.
- **DNA Solubilisation:** DNA is soluble in aqueous solution.
- **Contamination removal:** Proteins, RNA, and other macromolecules are eliminated by chemical and enzymatic methods.

- **DNA Precipitation:** Ethanol or isopropanol are commonly used for precipitating DNA.

➢ **Methods of DNA Extraction:** There are various proven techniques for DNA extraction, such as:

- **Organic Extraction (Phenol-Chloroform Method)**: A traditional approach for DNA extraction, the organic extraction method—also called the phenol–chloroform method—uses organic solvents to remove DNA from cellular components. This technique effectively denatures and removes proteins from a combination of phenol, chloroform, and isoamyl alcohol, therefore separating DNA from other cellular waste. Usually added to the lysed cell solution, the mixture is centrifuged to produce two separate phases: an organic phase including proteins and lipids and an aqueous phase including the DNA. After that, the DNA is extracted from the aqueous phase very deliberately. Although this approach produces high-quality DNA suitable for several downstream uses, it is labor-intensive and time-consuming and requires cautious management of dangerous chemicals between several phases.
- **Nonorganic techniques of DNA extraction**: These are commonly employed due to their ease, safety, and efficacy in avoiding toxic chemicals. The salting out method is a popular method that uses high concentrations of salt typically sodium chloride or ammonium acetate to precipitate proteins from cell lysate. The DNA remains in solution and is easily obtained with alcohol precipitation once the proteins have separated. Another effective approach in which the enzyme Proteinase K breaks down cellular proteins, including histones and other DNA-binding proteins, therefore releasing the DNA is the Proteinase K therapy. This process is effective and commonly used in combination with other steps to improve purity and yield. Both procedures are appropriate for general laboratory use as they are less hazardous and more user-friendly than organic extraction methods.
- **Silica-Based Adsorption Techniques:** Adsorption methods, particularly silica-based methods, are widely employed for DNA purification due to their efficiency and ease of use. This method drives DNA to adhere preferentially to a silica gel membrane or silica-coated beads under certain pH and salt concentration. The attached DNA is subsequently purified by washing away impurities and eluting in a low-salt buffer or water. Centrifugation or magnetic separation can be employed for

purification, depending on the system. Many commercial DNA extraction kits are built around these very dependable techniques.

- **Other Techniques:** Apart from silica-based approaches, numerous additional technologies are also applied for DNA separation. Using magnetic beads coated with DNA-binding molecules such as antibodies or silica, which allow the selective capture of DNA under a magnetic field for magnetic separation for automated, high-throughput processing especially, this method is quite useful. Anion exchange technique takes use of DNA molecules' negative charge, allowing them to adhere to positively charged resins while other biological components wash away. Ultimately, a traditional but labor-intensive method called cesium chloride (CsCl) density gradient centrifugation separates DNA according to buoyant density. Although it is less often employed nowadays, it produces highly purified DNA, which is particularly useful in research applications that need ultra-pure nucleic acids.

➢ **Evaluating DNA Quality and Yield:** Usually, the degree and quality of the extracted DNA are evaluated using:

- Spectrophotometry (Qubit) uses absorbance at 260 nm to estimate DNA concentrations. A260/A280 ratio of around 1.8 is desirable for clean DNA, whereas a lower ratio suggests protein contamination.
- Gel electrophoresis enables visualisation of DNA integrity and distribution.

➢ **Reagents Used for DNA Extraction**

- **Phenol-Chloroform Method:**

  ○ Lysis Buffer ○ NaCl (150 mM) ○ Tris-HCl 50 mM) ○ EDTA (10 mM) ○ Sodium Dodecyl Sulfate (20%) ○ Proteinase K (25 mg/ml) ○ Phenol, Chloroform, Isoamyl Alcohol (25:24:1) ○ 3M Sodium Acetate ○ 60% Isopropyl Alcohol ○ Ethanol ○ Tris-EDTA Buffer (pH 7.5)

- **Gu-HCl Method for Scat DNA Isolation:** ○ Lysis Buffer ○ Guanidium Hydrochloride (GuHCl, 6M) ○ Wash Buffer (Tris-Cl, EDTA, NaCl, 50% Ethanol) ○ Silica Beads

➢ **DNA Extraction Protocol**

- **Guanidium Hydrochloride (GuHCl) Method**

  The Guanidium Hydrochloride (GuHCl) method is a useful approach for extracting DNA from biological materials, particularly those containing large quantities of

protein, lipids, or RNA. The technique uses guanidine hydrochloride, a potent chaotropic drug that denatures proteins and disturbs cellular structures, therefore helping to isolate DNA.

- **Principles of the GuHCl Method**

  Guanidium hydrochloride (GuHCl) is a strong chaotrope that breaks the hydrogen bonds and hydrophobic interactions that hold proteins together, causing them to denature and detach from DNA. GuHCl also facilitates the lyse of cell membranes and dissolution of cellular debris, therefore releasing DNA. It also helps inactivate nucleases that could break down DNA. This produces the effective separation of DNA from many biological sources, including cell cultures, blood, and tissues.

- **Guanidium Hydrochloride (GuHCl) Method Procedure**

✞ Add 1 ml of EDTA to scat sample.

✞ Vortex for 30 minutes.

✞ Centrifuge at 10,000 rpm for 5 minutes, discard supernatant.

✞ Add 600 µl of 5M GuHCl.

✞ Add 30 µl of Proteinase K and vortex for 3-4 minutes.

✞ Incubate in water bath at 56°C overnight.

✞ Centrifuge at 10,000 rpm for 5 minutes, transfer supernatant to fresh vial.

✞ Repeat step 4 with same volume of GuHCl.

✞ Add 0.5 ml 2X wash buffer (heat shock method), vortex, and store at room temperature for 23 hours.

✞ Centrifuge at 10,000 rpm for 5 minutes, discard supernatant.

✞ Wash with 500 µl of 2X wash buffer, centrifuge at 10,000 rpm for 5 minutes.

✞ Add 500 µl of 80% ethanol, centrifuge at 10,000 rpm for 5 minutes.

✞ Dry the sample at 58°C using a block heater.

✞ Add 60 µl AE buffer, vortex, and centrifuge at 10,000 rpm for 5 minutes.

✞ Transfer clear supernatant to 1.5 ml tube, store at -20°C.

- **Qiagen Kit DNA Extraction Method**

- **Cell Lysis:** Mix 200 µl of ATL buffer with 20 µl of Proteinase K (50–80 mg) in a tissue sample. Vortex and incubate at 56°C overnight.
- **DNA Release:** Vortex 200 µl of buffer AL, then incubate for 15 minutes at 72°C. Add 200 µl ethanol.
- **DNA Binding:** Put the mixture in a spin column and centrifuge it for one minute at 8,000 rpm. Eliminate the filtrate.
- **DNA Washing:** Centrifuge at 8,000 rpm for 1 minute after adding 500 µl of buffer AW1. Eliminate the filtrate. Repeat using buffer AW2, run one minute at 8,000 rpm. Centrifuge for three minutes at 14,000 rpm.
- **DNA Elution:** Add 40 µl of AE buffer into the spin column, leave it at room temperature for a minute, and then centrifuge it for a minute at 8,000 rpm.

• **Phenol: Chloroform Method of DNA Isolation**

- **Sample Preparation:** Cut 80-100 mg of tissue into tiny pieces and place in a 1.5 ml Eppendorf tube.
- **Cell Lysis:** Add 25 µl Proteinase K, 60 µl 20% SDS, and 600 µl lysis buffer. Placed overnight at 56°C.
- **Phase Separation:** Before centrifuging for 10 minutes at 10,000 rpm, add an equivalent amount of saturated phenol and shake for 10 minutes.
- **Protein Removal:** Add 300 µl of phenol and 300 µl of chloroform:isoamyl alcohol (24:1). After mixing, centrifuge at 10,000 rpm for 10 minutes.
- **DNA Precipitation:** Add 600 µl of isoamyl alcohol and chloroform, centrifuge, and gather the sediment. Add 60% isopropyl alcohol along with 0.1 volume sodium acetate. To precipitate the DNA, incubate for one hour at room temperature.
- **Washing:** Use 70% ethanol to wash after centrifuging for 10 minutes at 10,000 rpm and discarding the supernatant. Continue with with 100% ethanol.
- **Resuspension:** Use Tris-EDTA buffer to dissolve the DNA.

1.13.2 PCR amplification

Polymerase Chain Reaction (PCR) is a commonly used molecular biology method for amplifying a particular part of DNA in vitro. The PCR technique is extremely efficient, enabling the amplification of a single DNA molecule into billions of copies. The reaction takes place in a thermocycler following these stages: The main steps of PCR amplification are:

- **Denaturation:** To separate the two strands, the double-stranded DNA (dsDNA) template is heated to temperatures between 92 and 95°C. This mechanism disrupts the hydrogen bonds between complementary bases, resulting in two single-stranded DNA molecules.
- **Annealing:** Short DNA primers can attach (anneal) to complementary sequences on the single-stranded template DNA by lowering the temperature to between 50 and 70°C. These primers are necessary to start the synthesis of DNA.
- **Extension:** To enable the thermostable enzyme Taq polymerase to create additional DNA strands, the temperature is increased to around 72°C. Taq polymerase stretches the primers using the free nucleotides in the reaction mixture (dNTPs), therefore producing new DNA strands complementary to the template. Repeated for 30 to 40 cycles, these three procedures rapidly increase the DNA segment of interest.

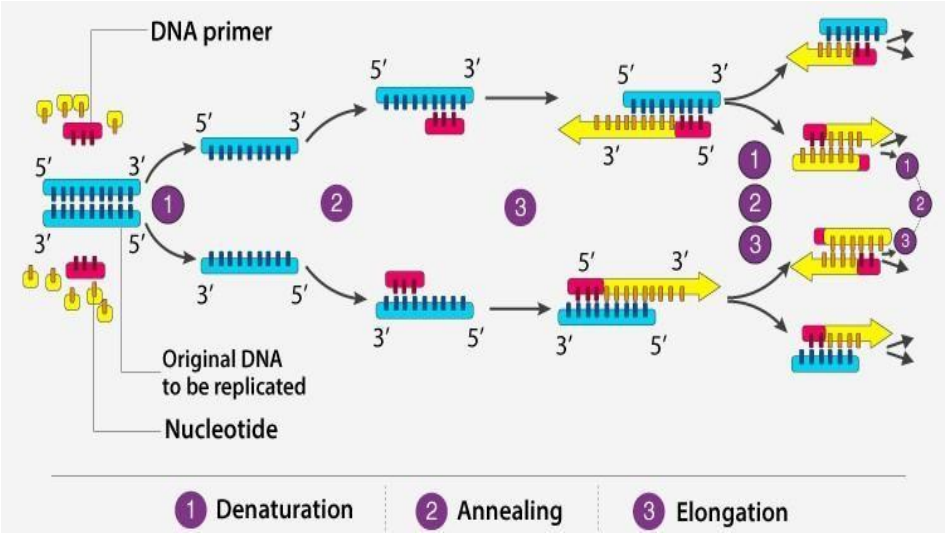

Fig. 1.3 PCR Amplification

- **Key PCR components**

- **Magnesium chloride ($MgCl_2$):** It acts as a cofactor for the Taq polymerase enzyme.
- **PCR Buffer (pH 8.3–8.8):** Provides the best conditions for the PCR procedure.
- **Deoxynucleoside Triphosphates (dNTPs):** dATP, dTTP, dCTP, and dGTP are the building blocks required to create new DNA strands.
- **Primers:** Short single-stranded DNA sequences (18-25 nucleotides) that bind to the target DNA region.
- **Target DNA:** The sequence to be replicated in DNA.

- **Taq Polymerase:** It is a thermostable DNA polymerase enzyme that extends primers and synthesises new DNA strands.

➢ **Types of PCR Techniques:**

- **Conventional (Qualitative) PCR:** This is the most basic type of PCR that amplifies a particular DNA sequence for qualitative analysis.
- **Multiplex PCR:** This technique enables the simultaneous study of numerous DNA segments by amplifying multiple targets (different DNA sequences) in a single reaction.
- **Nested PCR:** A modified PCR method that uses two rounds of amplification to increase specificity. The first PCR amplifies a larger area, but the second PCR employs internal primers to amplify a smaller, more targeted DNA fragment.
- **Reverse Transcriptase PCR (RT-PCR):** It is used to amplify cDNA (complementary DNA) generated from RNA. This approach is useful for analysing gene expression since it uses reverse transcriptase to convert mRNA into cDNA.
- **Quantitative PCR (qPCR):** It is also known as Real-Time PCR, measures the quantity of DNA or RNA in samples. Target DNA may be quantified using this method, which tracks DNA amplification in real time using fluorescent dyes or probes.
- **Hot-Start PCR:** This method lowers nonspecific DNA amplification by preventing DNA polymerase activity until the reaction reaches the high-temperature denaturation stage. This is accomplished by modified polymerases or antibodies indicated to prevent the polymerase from functioning at lower temperatures.

➢ **PCR Protocols:**

- **Normal Taq PCR Protocol:** This is the standard method for amplification of DNA using Taq polymerase.

○ **Reagents**:

✞ **Buffer**: 1 µL

✞ **dNTPs**: 0.5 µL

✞ **Primer F**: 0.30 µL (Forward Primer)

✞ **Primer R**: 0.30 µL (Reverse Primer)

✞ **Taq Polymerase**: 0.20 µL

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- **Water (H_2O)**: 6.75 µL
- **Total Volume**: 9.05 µL per sample/tube

○ **Procedure**:

- Mix all reagents in a 2 mL tube.
- Vortex the mixture and perform a short spin.
- Add 9 µL of the mixture per PCR tube.
- Add template DNA to each tube.
- Perform a short spin to ensure the DNA is mixed thoroughly with the reaction mixture.
- Run the PCR reaction in a thermocycler using standard PCR conditions.

• **Multiplex PCR** (for amplifying multiple targets in one reaction):

○

Reagents:

- **Q**

solution

: 1 µL

- **Multiplex PCR mix**: 4 µL
- **Primer F**: 0.25 µL (Forward Primer)
- **Primer R**: 0.25 µL (Reverse Primer)
- **Water (H_2O)**: 3.5 µL
- **Total Volume**: 9.0 µL per sample/tube ○ **Procedure**:
- Combine all reagents in a 2 mL tube.
- Vortex and short spin.
- Add 9 µL of the mixture per PCR tube.
- Add template DNA to each tube.
- Perform a short spin.
- Run the PCR reaction in a thermocycler

1.13.2 DNA Sequencing

DNA sequencing is the technique of determining the precise order of nucleotides [adenine (A), thymine (T), cytosine (C), and guanine (G)] in a DNA molecule. It reveals genetic information essential for understanding biology, evolution, and illness, therefore offering an orientation trace of life.

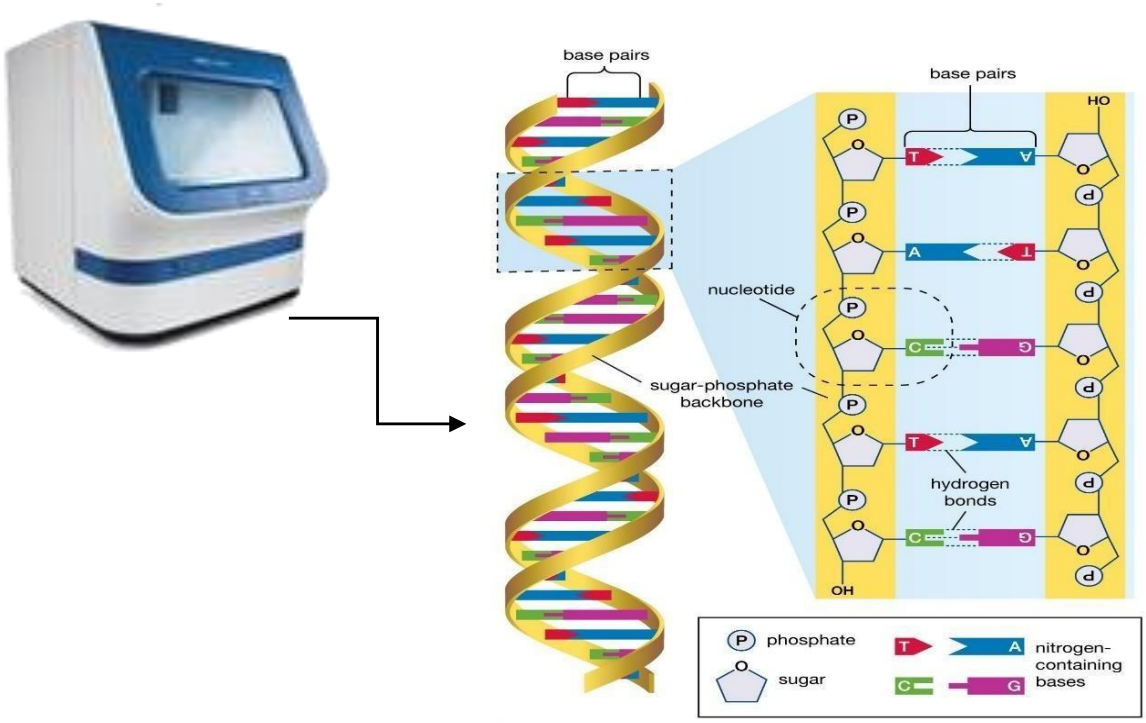

Fig. 1.4 DNA Sequencing

➢ **Various DNA Sequencing Methods**

- **Sanger Sequencing:** Sanger sequencing, also known as the chain termination method, was the first widely used technology for DNA sequencing and is still the gold standard for short DNA fragments. It depends on chain-terminating di-deoxynucleotides (ddNTPs) selectively being incorporated during DNA replication. Every ddNTP is tagged with a fluorescent dye, which allows capillary electrophoresis read the sequence. Sanger sequencing is low through put and best suited for sequencing individual genes or limited genomic areas even if it is quite precise. It is frequently utilised in clinical settings for diagnosis or confirmation of next-generation sequencing findings.
- **NGS, or next-generation sequencing:** Next-generation sequencing transformed genomic research by allowing for the simultaneous sequencing of millions of DNA fragments. Unlike Sanger sequencing, NGS methods execute sequencing in parallel,

•

resulting in huge volumes of data in a short period of time and at a reduced cost per base. Sequencing-by-synthesis platforms, such as Illumina, employ fluorescent signals to identify nucleotides as they are added one at a time. NGS is widely utilised for whole-genome sequencing, transcriptome analysis (RNA-Seq), epigenomics, and clinical diagnostics, providing greater understanding of genetic diversity, gene expression, and disease processes.

- **Pyrosequential sequencing:** Pyrosequencing is a technique that detects pyrophosphate release during nucleotide incorporation. It was originally quite common for some genotyping uses and short-read sequencing. Faster than Sanger sequencing, it has limits in read length and homopolymer accuracy and has mainly been supplanted by more sophisticated NGS technology. However, it is still used in niche applications such as microbial community analysis and methylation investigations.
- **Massively Parallel Signature Sequencing (MPSS):** MPSS was an early kind of high throughput sequencing. It consisted on amplifying DNA segments attached to microbeads, then hybridising and enzymatic cleaving the resulting sequence. MPSS assisted in paving the path for massive transcriptome profiling and helped create more polished NGS platforms even if it is not yet extensively used.
- **Ligated sequencing (e.g., SOLiD):** Another technique of DNA sequencing utilising short fluorescently labelled oligonucleotides binding to a complementary DNA strand is ligation. The SOLiD platform, developed by Life Technologies, is an example of this method.

  Through dual-base encoding, it provides great accuracy; yet, its complicated procedures have eroded appeal as simpler, more effective NGS methods have emerged.

1.13.4 Gel Electrophoresis

Gel electrophoresis is a method for separating DNA, RNA, and proteins according to their size and charge. In the case of nucleic acids, agarose gel electrophoresis is the most popular method. When exposed to an electric field, DNA becomes negatively charged and migrates towards the positive electrode (anode). The gel functions as a molecular filter, allowing smaller fragments to flow more quickly than bigger ones.

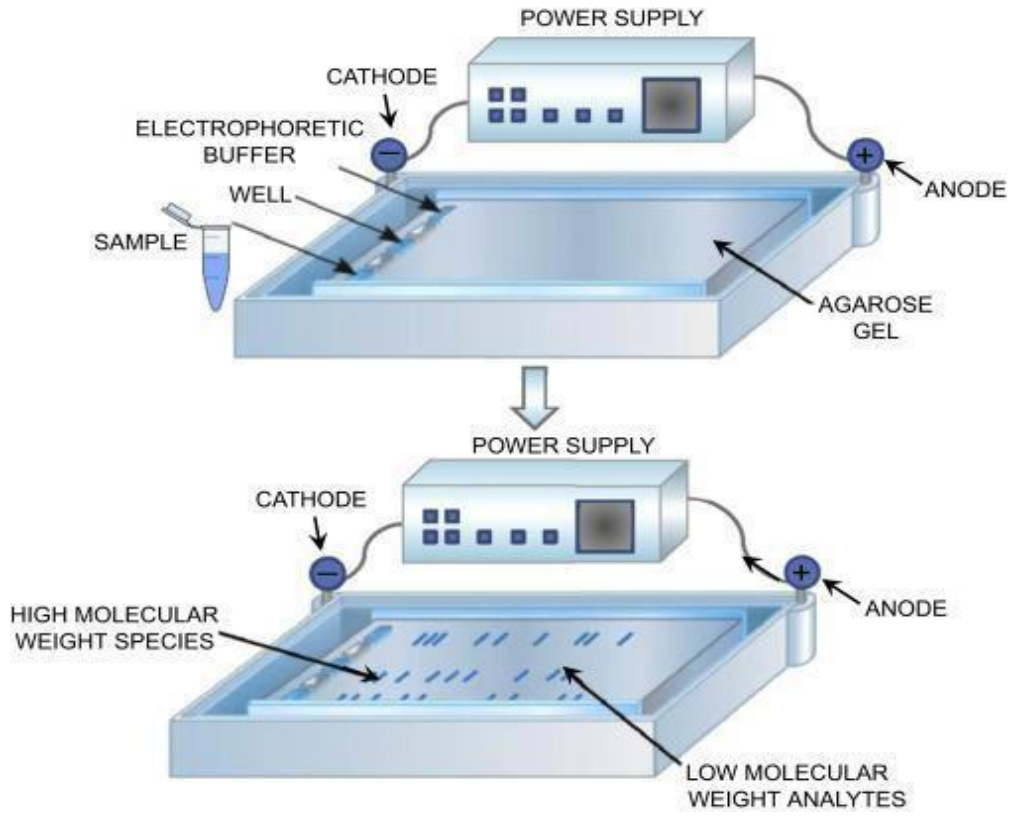

Fig. 1.5 Gel Electrophoresis

- **Agarose Gel Electrophoresis**

  This approach is commonly used for analysing DNA fragments, particularly following DNA extraction, restriction digestion, or PCR amplification. The components, preparation, and step-by-step technique are outlined below.

❖ **Materials Required**

○ Casting tray ○ Gel cassette ○ Comb ○ 10 µL micropipette and tips ○ Conical flask ○ Measuring cylinder ○ Agarose powder ○ 1X TAE (Tris-Acetate-EDTA) buffer ○ Ethidium Bromide (EtBr) – for DNA staining ○ Microwave or heating oven

❖ **Procedure: Agarose gel electrophoresis**

The agarose gel electrophoresis technique begins with a 0.8% agarose gel made for DNA sample analysis following extraction and isolation. First, 100 mL of 1X TAE (Tris-AcetateEDTA) buffer is added to a conical flask. Then, 0.8 gms of agarose powder are precisely weighed and added to the buffer. After careful swirling, the mixture is heated in a microwave or oven for about one minute, or until the agarose has totally dissolved and the solution turns clear. After cooling to 50-60°C, add 4 µL

- of ethidium bromide (EtBr) to stain the DNA for UV visualisation. After that, the ready gel solution is placed undisturbed at room temperature for around 15 to 20 minutes, then poured into a casting tray with a comb set to create wells.

  In order to resolve smaller DNA fragments for DNA analysis following polymerase chain reaction (PCR), a higher 2% gel is needed. In this scenario, using the same heating and staining techniques, two gms of agarose are dissolved in one hundred milliliters of 1X TAE buffer. Then, the gel will be placed in the electrophoresis chamber—filled with 1X TAE running buffer—once it has set. Mixed with loading dye, DNA samples are pipetted into the wells using special care; a molecular weight marker or DNA ladder is loaded into a separate well for size reference. The gel is then operated for around thirty to forty-five minutes at an 80–120-volt voltage. DNA fragments move across the gel matrix towards the positive electrode during electrophoresis, smaller fragments migrating faster than bigger ones. The gel is visualised on a UV transilluminator once the run is over. Under UV light, the intercalated ethidium bromide fluoresces to expose the separated DNA bands, which may then be seen using gel documentation systems.

Chapter-2

To assess the evolutionary relationships of different bharal population

2.1 Background

The Bharal (*Pseudois nayaur*) is a wild caprine inhabiting the rugged mountainous terrains of the Himalayas. Its distribution spans India, Nepal, Bhutan, China, and northern Pakistan, and its ecological and taxonomic significance has been well documented (Martens, 2011). The species is currently listed as *Least Concern* by the International Union for Conservation of Nature (IUCN), yet it is afforded the highest level of legal protection under Schedule I of the Indian Wildlife (Protection) Act, 1972, and is included in Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Despite this wide distribution and legal protection, substantial gaps remain in the evolutionary understanding of bharal populations across their range.

The taxonomic classification of bharal has long been debated, largely due to the morphological and genetic variability observed across its distribution. The genus *Pseudois* comprises two recognised species: the dwarf Bharal (*P. schaeferi*) (Zeng et al., 2008) and the Bharal (*P. nayaur*). Within *P. nayaur*, putative subspecies—*P. n. nayaur* in the western Himalayas and *P. n. szechuanensis* in the eastern Himalayas—further contribute to taxonomic uncertainty (Saini et al., 2025). Additional ambiguity arises from conflicting views on the taxonomic status of certain populations: some authors regard the dwarf Bharal as a subpopulation of *P. n. nayaur*, whereas others propose the Helan Mountain population *in* China as a distinct subspecies, *P. n. helanshanensis*. These discrepancies underscore the need for comprehensive phylogenetic analyses to resolve the genetic structure and evolutionary history of bharal.

Mitochondrial DNA (mtDNA) analyses have proven highly effective for inferring evolutionary relationships and assessing population genetics in wild ungulates. Previous studies using mtDNA markers—particularly *cytochrome b*—have identified distinct lineages among Bharal populations (Sodhi, 2022). However, whole-mitogenome approaches remain underexplored, especially for bharal in India.

Given the species' extensive distribution across the Indian Himalayan Region (IHR), further research is needed to quantify genetic diversity and evaluate the influence of habitat connectivity on population structure (Yadav et al., 2020).

Beyond phylogenetic classification, bharal holds significant ecological importance. As a primary herbivore in trans-Himalayan ecosystems, it plays a key role in maintaining ecological balance (Bagchi, 2009). Its diet primarily consists of alpine meadow vegetation, supplemented by shrubs and forbs during resource-scarce periods. Bharal also forms a crucial prey base for apex predators such as the Himalayan wolf (*Canis lupus*) and snow leopard (*Panthera uncia*), meaning fluctuations in its population can directly impact predator conservation (Wolf, 2016). Nevertheless, the species faces increasing threats from habitat fragmentation, climate change, and human disturbances challenges that demand conservation strategies informed by both ecological and genetic evidence.

This chapter aims to employ complete mitochondrial genome sequencing to:

1. Clarify phylogenetic relationships between bharal and other bovids, thereby addressing taxonomic uncertainties.
2. Assess the genetic diversity of Indian bharal populations.
3. Identify potential evolutionary lineages by comparing Indian bharal with closely related species.

The findings will contribute to a clearer understanding of bharal's evolutionary history and provide an empirical basis for conservation planning for Himalayan wildlife.

2.2 Methodology

Study Area

The biological samples of Bharal (*Pseudois nayaur*) collected from the eastern and western Himalayan areas to evaluate the phylogenetic relationships among geographically diverse populations (Fig 2.1).

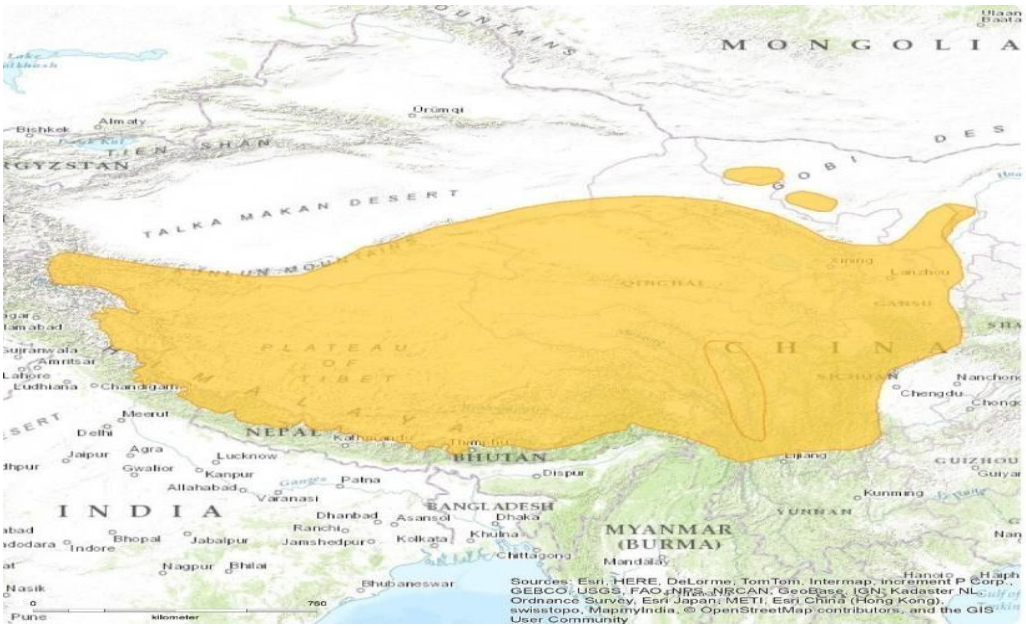

Figure 2.1. Distribution map of bharal (*Pseudois nayaur*) (IUCN, Harris 2014).

- **Sample Collection and DNA Isolation**

This study examined a total of 35 fecal samples and 2 Tissue samples of *Pseudois nayaur* for complete mitogenome were obtained from verified specimens in partnership with local wildlife officials in various parts of the Indian Himalayas (fig 2.2 & table 2.1). One sample is collected from illumine and other one is obtained from the given primer sequence (table 2.2). Both samples were procured from deceased individuals to adhere to ethical norms; hence, permission from the Animal Ethics Committee was unnecessary. Genomic DNA (gDNA) was extracted utilizing a modified DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) for tissue and hair specimens. A Gu-HCl based silica-binding technique was employed for antlers or bone samples (Gupta et al., 2013).

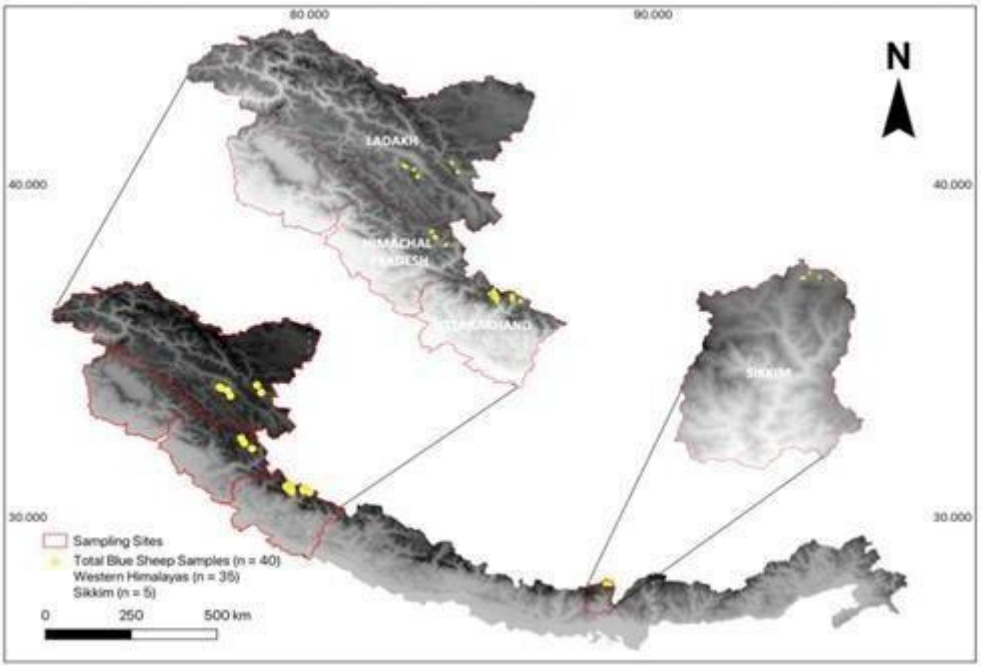

Fig. 2.2 A map showing the distribution and sampling locations for the current study. Yellow circles indicate the location of the sampling sites

Table 2.1. Details of *Pseudois nayaur* samples used for complete mitochondrial genome analysis

Sample Type	**Number of Samples**	**Collection Source / Method**	**Remarks**
Fecal samples	35	Collected from verified specimens in collaboration with local wildlife officials across different regions of the Indian Himalayas	Utilized for complete mitochondrial genome sequencing
Tissue samples	2	Obtained from verified specimens in collaboration with local wildlife officials	One sample sequenced using Illumina platform; the other generated using primer-based amplification (see Table 2.2)

Table 2.2: Details of the primers used in this study to sequence mitogenome Bharal

Primer Name	Primer Sequence
DLU405	5'-ACCATGCCGCGTGAAACCAGCA-3'
12SL41	5'-GYGYGGATRCTTGCATGTGTA-3'
U1230	5'-CACTGAAAATGCCTAGATGAG-3'
L2226	5'-CTAGGTGTAAACTAGRTGCTT-3'
12SU829	5'-GCACGCACACACCGCCCGTCAC-3'
16SL518	5'-CGCTTTCTTAATTGRTGGCTGC-3'
16SU365	5'-AGCCTGGTGATAGCTGGTTGTCC-3'
16SL1056	5'-AAGCTCCATAGGGTCTTCTCGTC-3'
16SU946	5'-CCGTGCAAAGGTAGCATAATCA-3'
N1L64	5'-CCTAGNACTTTTCGTTCNACTA-3'
Uleu	5'-GTGGCAGAGCCCGGTAATTG-3'
IleL	5'-TTACTCTATCAAAGTAACTC-3'
N1U840	5'-TYCGAGCATCHTAYCCHCGATT-3'
N2L492	5'-TGGTTTAGBCCBCCTCAKCCYCC-3'
N2U354	5'-CACTTYTGAGTNCCAGAAGT-3'
AsnL	5'-TAGGGTRTTTAGCTGTTAAC-3'
TrpU	5'-AGACCAAGAGCCTTCAAAGC-3'
C1L339	5'-GCTTCWACTATDGADGATGC-3'
C1U246	5'-GGNGGNTTYGGHAAYTGACT-3'
C1L1017	5'-GAARATRAAGCCTAGRGCTCA-3'
C1U897	5'-TTYACHGTHGGAATAGAYGT-3'
C2L15	5'-GCRTCTTGRAANCCTARTTG-3'
SerU	5'-CCCCCYAYWRYTGGTTTCAAGCCA-3'
A8L1	5'-GTKGAYGTRTCTAGTTGYGGCAT-3'
C2U603	5'-CAATGCTCHGARATYTGYGG-3'
C3L45	5'-GANARDGCTCCYGTDAGNGGTCA-3'
A6U654	5'-GCCTAYGTNTTYACYCTNCTAGT-3'

GlyL	5'-TGATTGGAAGTCARYTGTAC-3'
C3U780	5'-GTHTCYATCTATTGATGAGG-3'
N4L27	5'-CAGGTYAGRGGDATDAGTAT-3'
U213M1	5'-AGCYTGYGAAGCAGCACTAGG-3'
L918M1	5'-GCKGTRGCTCCTATRTARCTTCA-3'
N4U840	5'-AGCTCHATYTGYYTHCGYCAAAC-3'
Leu2L	5'-CCAATTTTTTGGYTCCTAAGRCC-3'
Ser2U	5'-CCGAAAAAGYAYGCAAGAACTGC-3'
N5L652	5'-GCDGATTTTCCDGTKGCDGCTA-3'
N5U501	5'-GACGARCAGAYGCHAAYACAGC-3'
N5L1214	5'-GTDAKTADDAGGGCTCAGGCG-3'
N5U1146	5'-GGMAGCCTNGCNYTAACAGG-3'
N6RL154	5'-AGTTTAATGGDHTDGGDGATTG-3'
N6RU102	5'-CCATAACTRTAYAAAGCHGCAA-3'
CBL402	5'-CCTCARAATGATATTTGKCCTCA-3'
CBU162	5'-CAGGMCTATTCCTRGCHATACA-3'
LTHR	5'-CCCTTYTCTGGTTTACAAGACC-3'
U1068	5'-CATCGGACAACTAGCATCTAT-3'
L482	5'-CCTGAAGWAAGAACCAGATG-3'

- **PCR Amplification and Sequencing of the Mitogenome**

  Polymerase Chain Reaction (PCR) amplification was performed in 20 μL reaction volumes comprising 10–20 ng of template gDNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). Amplification was conducted using 23 overlapping pieces that included the whole mitochondrial genome, including areas that encode cytochrome b and cytochrome c oxidase I (COI) for enhanced coverage. The PCR cycling protocol comprised an initial denaturation at 95°C for 5 minutes, succeeded by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 54–56°C for 40 seconds, extension at 72°C for 50 seconds, and a final extension at 72°C for 15 minutes.

PCR products were validated using 2% agarose gel electrophoresis, identified under UV illumination with ethidium bromide, and purified using Exonuclease-I and Shrimp Alkaline Phosphatase methods. BigDye Terminator v3.1 chemistry was used bidirectionally on an ABI 3500XL Genetic Analyzer (Applied Biosystems, USA).

- **Complete Mitogenome Assembly, Annotation, and Characterisation**

This study examined a total of two mitochondrial DNA samples. Overlapping DNA segments were included into the whole mitochondrial genome using Sequencher® v5.4.6. Annotation was performed using the MITOS WebServer (Bernt et al., 2013), and gene mapping was shown with the Organellar Genome DRAW tool (Lohse et al., 2013). The base composition, AT/GC skew, and structural features (including intergenic spacers and overlapping regions) were manually calculated and validated using MEGA X (Kumar et al., 2018).

- **Complete Mitogenome Phylogenetic Analysis**

To evaluate the evolutionary status of *Pseudois nayaur*, comprehensive mitochondrial genome sequences of relevant Bovidae species were retrieved from GenBank. Mitogenomes of *Bos taurus*, *Bos javanicus*, *Capra hircus*, *Ovis aries*, among others, were included. Furthermore, *Moschus moschiferus* served as an outgroup. Bayesian inference phylogenetic analysis was performed with BEAST v1.7 (Drummond et al., 2012). MCMC simulations were conducted using four chains over 10 million generations, with sampling occurring every 100 generations, and the initial 5,000 generations discarded as burn-in. The consensus trees obtained were shown utilizing FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

- **Phylogeography of *Pseudois nayaur***

The phylogeographic structure of Bharal was examined to analyze the geographic distribution and genetic divergence across populations in India and China. A median-joining (MJ) network was generated using PopART software to visually represent the relationships among different mitochondrial haplotypes. This network result indicated the grouping of unique genetic variants and find if they matched geographical locations. The results exposed a significant genetic difference between the separate haplogroups of *P. n. nayaur* in the western Himalayas and *P. n. szechuanensis* in the eastern Himalayas. Although the complete mitogenome

sequence of Indian (*P. nayaur* India 01) was found to be genetically distinct, the Chinese sequences grouped tightly and indicated little gene flow and maybe historical isolation between these two lineages. These results suggest that bharal populations could have undergone region-specific evolutionary processes molded by physical and ecological constraints.

2.3 Mitochondrial DNA Haplotype Diversity of *Pseudois nayaur* Based on Control Region and Cytochrome *b* Gene Sequences Across Different Geographic Regions

S.No.	Accession no.	Origin	Haplotype
1	PP187055	Uttarakhand	**1**
2	PP187056, PP187057, PP187059	Uttarakhand	**2**
3	PP187058	Uttarakhand	**3**
4	PP187065	Uttarakhand	**4**
5	PP187060	Uttarakhand	**5**
6	PP187061	Uttarakhand	**6**
7	PP187062, PP187063	Uttarakhand	**7**
8	PP187064	Uttarakhand	**8**
9	PP187066, PP187070	Himachal Pradesh	**9**
10	PP187067, PP187071, PP187072, PP187074	Ladakh	**10**
11	PP187068, PP187069	Ladakh	**11**
12	PP187073	Ladakh	**12**
13	PQ583502	Uttarakhand	**13**
14	PP187076, PP187077, PP187078, PP187079	Himachal Pradesh	**14**
15	PP187080, PP187084, PP187085	Uttarakhand	**15**
16	PP187075	Uttarakhand	**16**
17	PP187082, PP187087	Uttarakhand	**17**
18	PP187081	Uttarakhand	**18**
19	PP187083, PP187086, PP187088	Uttarakhand and Ladakh	**19**

20	PP187089, PP187090	Sikkim	**20**
21	PP187091	Sikkim	**21**
22	PP187092	Sikkim	**22**
23	PP187093	Sikkim	**23**
24	EF420241.1	China	**24**
25	EF420240.1	China	**25**
26	KX641001.1	China	**26**
27	DQ234685.1	China	**27**
28	DQ234684.1	China	**28**
29	DQ234683.1	China	**29**
30	DQ234682.1	China	**30**

S.No.	Accession no.	Origin	Haplotype
1	PP348784, PP348785, PP348786, PP348787, PP348788, PP348789, PP348790, PP348794, PP348795, PP348798, PP348801	Ladakh and Himachal Pradesh	**1**
2	PP348791, PP348793, PP348796, PP348797	Uttrakhand	**2**
3	PP348792	Himachal Pradesh	**3**
4	PP348799, PP348800	Himachal Pradesh	**4**
5	PP348802	Uttrakhand_India	**5**
6	PP348803	Uttrakhand_India	**6**
7	PP348804, PP348811	Uttrakhand_India	**7**
8	PP348805, PP348806	Uttrakhand_India	**8**
9	PP348807, PP348808	Uttrakhand_India	**9**
10	PP348809, PP348810	Uttrakhand_India	**10**
11	PP348812, PP348813	Uttrakhand_India	**11**
12	PP348814, PP348815	Uttrakhand_India	**12**

13	PP348816	Uttrakhand_India	**13**
14	PP348817	Uttrakhand_India	**14**
15	PP348818	Uttrakhand_India	**15**
16	PP348819	Ladakh	**16**
17	PP348820	Uttrakhand_India	**17**
18	JQ406563.1	China	**18**
19	JQ406560.1	China	**19**
20	JQ406559.1	China	**20**
21	ON412375.1	India	**21**
22	ON412376.1	India	**22**
23	ON412378.1	India	**23**
24	ON412379.1	India	**24**
25	ON412377.1	India	**25**
26	JQ406562.1	China	**26**
27	JQ406564.1	China	**27**
28	JQ406561.1	China	**28**
29	JQ406565.1	China	**29**
30	JQ406567.1	China	**30**
31	AF493575.1	China	**31**
32	JN839966.1	China	**32**
33	JN839981.1	China	**33**
34	JN839982.1	China	**34**

35	JN839975.1	China	**35**
36	JN839979.1	China	**36**
37	JN839973.1	China	**37**
38	JN839980.1	China	**38**

2.3 Results & Discussion

- **Mitogenome Organization of *Pseudois nayaur*** The whole mitogenome of the Bharal was obtained by Illumina sequencing and submitted to the NCBI GenBank under the accession number OP583799 (Fig. 2.3). It contained two ribosomal RNA genes, 22 transfer RNA genes, 13 protein-coding genes, and a non-coding regulatory area (D-loop region) (Fig. 2.3 and Table 2.3). The nucleotide composition of the mtDNA from Bharal was A (33.6%), T (26.3%), C (26.9%), and G (13.2%). Table 2.8. With the exception of the eight tRNA genes, the bulk of the genes were encoded on the H-strand (tRNAGln, tRNAAla, tRNAAsn, tRNACys, tRNATyr, tRNASer, tRNAGlu, tRNAPro). The regulatory region was located between tRNAPro and tRNAPhe (Table 2.4). We discovered eight pairs of overlapping genes: tRNAIle/tRNAGln, COI/tRNASer, ATP8/ATP6, ATP6/COIII, ND4L/ND4, ND5/ND6, and tRNAThr/tRNAPro. The minimal overlap was 1 bp between ATP6/COIII and tRNA-Thr/tRNA-Pro, whereas the maximal overlap was 40 bp between ATP8 and ATP6 (Table 2.4). Thirteen intergenic spacers between the mitochondrial areas were identified, varying in length from 1 to 32 base pairs. The most significant gap (32 bp) was noted between tRNAAsn and tRNACys (Table 2.4). The whole mitogenome of Bharal exhibited an AT bias of 60%, with 40% attributed to GC content. We calculated the values of AT-skew, GC-skew, AT%, and GC% to characterize the nucleotide compositions of the whole mitogenome. All examined Bharal species had a positive AT-skew (0.112), whilst the others displayed a negative GC-skew (0.341).

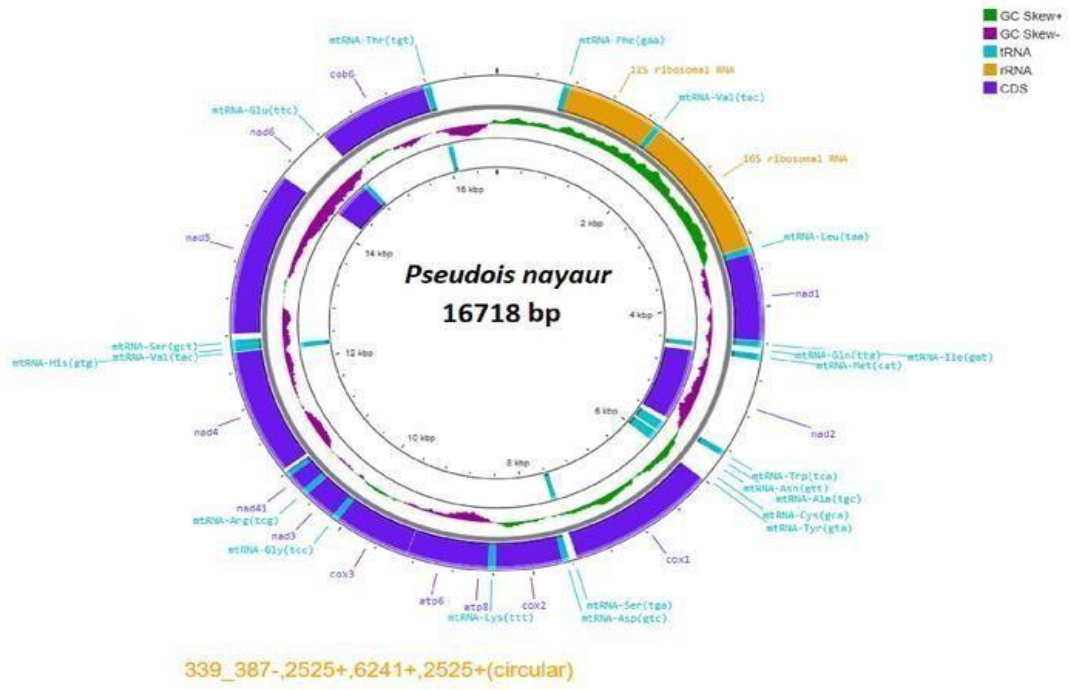

Figure 2.3. Illustration of the location of genes on the complete mitochondrial genome of *Pseudois nayaur* (16,718 bp).

Table 2.4: Organization of the complete mitochondrial genome of *Pseudois nayaur*

Feature	Start	End	Size	Start Codon	Stop Codon	Anti-codon	Strand	Space/ Overlap
tRNAPhe	1	68	68	–	–	GAA	H	0
12S rRNA	69	1025	957	–	–	–	H	0
tRNA-Val	1026	1092	67	–	–	TAC	H	0
16S rRNA	1093	2665	1573	–	–	–	H	0
tRN ALeu	2666	2740	75	–	–	TAA	H	0
ND1	2743	3698	956	ATG	TA–	–	H	2

tRNA-Ile	3699	3767	69	–	–	GAT	H	0
tRN AGln	3765	3836	72	–	–	TTG	L	-3
tRN AMet	3839	3907	69	–	–	CAT	H	2
ND2	3908	4949	10 42	ATA	T–	–	H	0
tRN A-Trp	4950	5016	67	–	–	TCA	H	0
tRN A-Ala	5018	5086	69	–	–	TGC	L	1
tRN AAsn	5088	5160	73	–	–	GTT	L	1
tRN ACys	5193	5260	68	–	–	GCA	L	32
tRN A-Tyr	5261	5328	68	–	–	GTA	L	0
COI	5330	6874	15 45	ATG	TAA	–	H	1
tRN A-Ser	6872	6942	71	–	–	TGA	L	-3
tRN AAsp	6948	7015	68	–	–	GTC	H	5
COII	7017	7700	684	ATG	TAA	–	H	1

tRN ALys	7704	7771	68	–	–	TTT	H	3
ATP8	7773	7973	201	ATG	TAA	–	H	1
ATP6	7934	8614	681	ATG	TAA	–	H	-40
COII	8614	9397	784	ATG	T–	–	H	-1
tRN AGly	9398	9466	69	–	–	TCC	H	0
ND3	9467	9812	346	ATA	T–	–	H	0
tRN AArg	9813	9882	70	–	–	TCG	H	0
ND4L	9883	10,179	297	ATG	TAA	–	H	0
ND4	10,173	11,873	1701	ATG	T–	–	H	-7
tRN A-His	11,551	11,619	69	–	–	GTG	H	0
tRN A-Ser	11,621	11,679	59	–	–	TGA	H	1

tRNA Leu	11,681	11,750	70	–	–	TAG	H	1
ND5	11,751	13,571	1821	ATA	T–	–	H	0
ND6	13,578	14,083	506	ATG	TAA	–	L	-17
tRNA Glu	14,093	14,161	69	–	–	TTC	L	9
CYTB	14,168	15,295	1128	ATG	AGA	–	H	4
tRNA-Thr	15,296	15,368	73	–	–	TGT	H	0
tRNA-Pro	15,368	15,428	61	–	–	TGG	L	0
Control region	15,434	16,718	1285	–	–	–	H	–

- **Protein-coding genes (PCGs)**

Protein-coding genes (PCGs) in the Bharal mitogenome measuring 11,319 bp in total length and including 64 bp overlapping sections, has 13 protein-coding genes (PCGs) that constitute 67.70% of the whole mitogenome. PCGs generally exhibit the

following basic compositions: A = 31.4%, T = 27.7%, G = 13.0%, and C = 27.9%. Table 2.8. PCGs often have a base composition of A = 31.4%. G constitutes 13.0%, T comprises 27.7%, and C accounts for 27.9%. The cytochrome c oxidases CO*I*, CO*II*, and CO*III*; the NADH dehydrogenases ND1, ND2, ND3, ND4, ND5, and ND4L; the ATPases ATP6 and ATP8; the cytochrome *b* gene (Cyt *b*); and the minority strand or L-strand gene (NADH dehydrogenase: ND6) comprise the 12-majority strand or Hstrand (Fig. 2.3 and Table 2.3). AT% occurred more frequently than GC% (59.1% compared to 40.9%). To clarify the nucleotide distribution in protein-coding genes, we assessed the base skews among several Bharal species. The average AT and GC skews values for Bharal PCGs were 0.062 and 0.364, respectively. The nucleotide composition was biased by cytosine, as seen by the negative GC skewness values. The ND5 gene, measuring 1821 bp, was the longest, while the ATP8 gene, at 201 bp, was the smallest among the 13 protein coding genes (PCGs). All 13 protein-coding genes started with either ATG or ATA. Among the 13 PCGs, we identified seven full stop codons (TAA), whereas ND1, ND2, ND3, and COIII utilized incomplete codons (TA- or T-). (Table 2.3). During mRNA maturation, a posttranscriptional addition of polyadenylation happened to finalize the protein-coding genes that were deficient in a full stop codon. The 13 protein-coding genes of Bharal have a relative synonymous codon use (RSCU) of 3773 codons, excluding stop codons (Fig. 2.4). Leucine (15.58%) and tryptophan (2.72%) were identified in Bharal PCGs at the highest and lowest rates, respectively (Fig. 2.5).

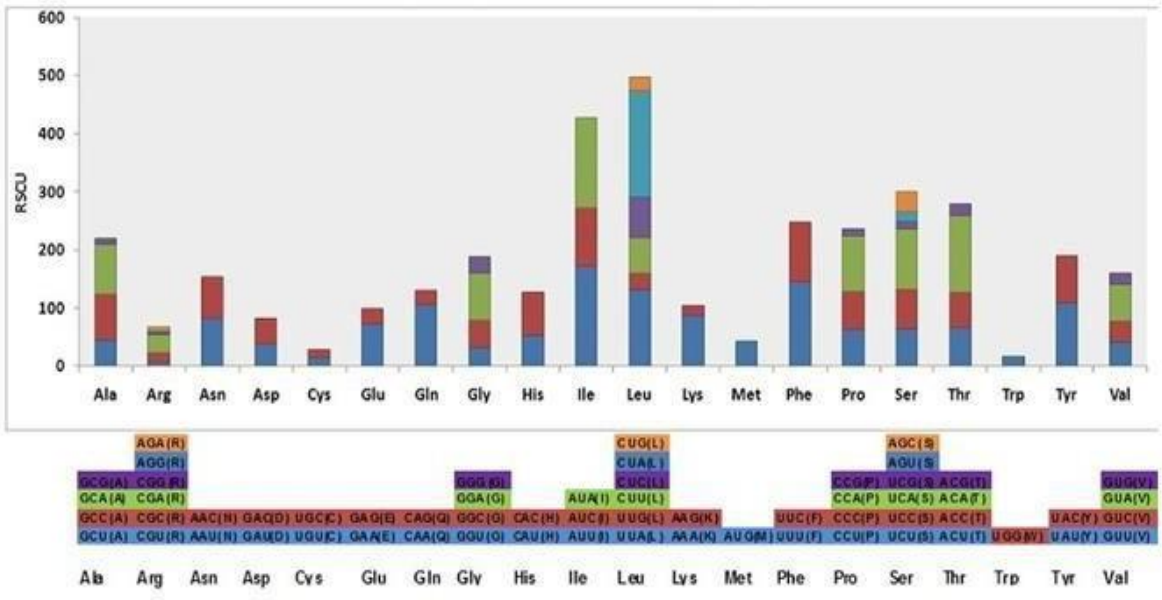

Figure 2.4. Relative synonymous codon usage (RSCU) of the mitochondrial protein coding genes *Pseudois nayaur* of the mitochondrial genome. Codon count numbers are provided on the X-axis.

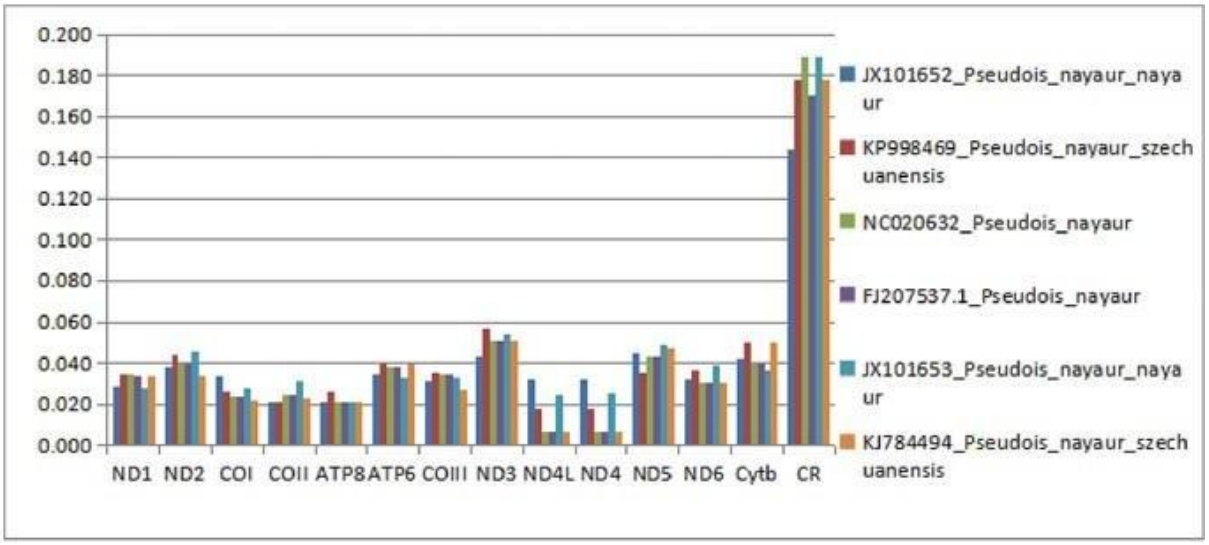

Figure 2.5. Comparative pairwise genetic distance in the protein-coding genes and control region of other subspecies *Pseudois nayaur*.

- **Genetic Distance within Bharal and Other Bovidae**

Table 2.5 shows the distance in genes matrix derived from the control region of mitochondrial DNA among Bharal groups reveals significant divergence patterns. The genetic distances (below the diagonal) indicate that the Western Himalayas exhibit considerable divergence from both the Eastern Himalayas (19%) and the China Mountains (22%), suggesting restricted gene flow and notable evolutionary isolation. The genetic distance between the Eastern Himalayas and the China Mountains is comparatively lower (5%), indicating a tighter genetic affinity between these two groups. The standard errors (above the diagonal) are comparatively low, with values of 0.0219 (between the Western and Eastern Himalayas), 0.0328 (between the Western Himalayas and China Mountains), and 0.0105 (between the Eastern Himalayas and China Mountains), indicating credible estimations. The data substantiates the presence of diverse genetic lineages among regional populations, notably a significant divergence of the Western Himalayan Bharal from others.

Table 2.5: Genetic distance of control region among Bharal (*Pseudois nayaur*) are presented (below the diagonal), and their standard errors (above the diagonal)

Location	**Western Himalayas**	**Eastern Himalayas**	**China Mountains**
Western Himalayas	-	0.0219	0.0328
Eastern Himalayas	0.1906	-	0.0105
China Mountains	0.2223	0.0546	-

The genetic distance (table 2.6) derived from the *cytochrome b* (*cyt b*) gene across Bharal (*Pseudois nayaur*) populations throughout several locations indicates differing levels of genetic divergence. The genetic distance in between the western Himalayan blue sheep and eastern Himalayan blue sheep is 3.9%, although the distance between the western Himalayas and the China Mountains is somewhat lower at 4%, suggesting a strong genetic affinity between these two locations. The genetic distances between the eastern Himalayas and the China Mountains (2.8%), and particularly between the western Himalayas and both the eastern Himalayas (3.9%) and the China Mountains (4%), indicate a considerably greater genetic divergence. The data suggest that groups in the eastern Himalayas and China Mountains have closer genetic affinity, but those from the western Himalayas are more genetically divergent, perhaps attributable to previous geographic isolation or varying evolutionary pressures. The standard errors, although not elaborated upon here, would further substantiate the statistical dependability of the measured distances.

Table 2.6: Genetic Distance of Cyt *b* among Bharal (*Pseudois nayaur*) are presented and their standard errors

Location	Western Himalayas	Eastern Himalayas	China Mountains
Western Himalayas	-	0.0115	0.011

Eastern Himalayas	0.0395	-	0.0086
China Mountains	0.0408	0.0288	-

The genetic differentiation matrix in Table 2.7 outlines the pairwise genetic distances among *Pseudois nayaur* (comprising *Pseudois nayaur* WII_01, *P. n. nayaur*, and *P. n. szechuanensis*) and other closely and distantly related ungulate species. In the *Pseudois* group, minimal genetic distances are noted, namely 2% between *P. n.* WII_01 and *P. n. nayaur*, and 4% between *P. n. nayaur* and *P. n. szechuanensis*, signifying tight evolutionary lineage within the species complex. Genetic distances between *Pseudois* species and domestic goat (*Capra hircus*), domestic sheep (*Ovis aries*), and *Capra ibex* vary from 8% to 10%, indicating intermediate divergence and a common ancestral branch within *Caprinae*. Significant divergence is noted among *Bovinae* subfamily members, including *Boselaphus, Bubalus bubalis, and Bos gaurus* (13%-14%), indicating their remote taxonomic affiliation. *Ovibos moschatus* and *Capricornis* thar *jamrachi* have intermediate divergence levels (8%–14%), consistent with their classification within *Caprini*, however external to the primary *Pseudois-Capra-Ovis* species. A minimum distance of 0.2% is noted between *Ovis aries* and *Ovis orientalis*, affirming their conspecific or closely related status. The greatest difference is observed between *Pseudois nayaur* and Sus scrofa (19%), highlighting the considerable evolutionary gap across artiodactyl groups. The matrix facilitates explicit taxonomic differentiation across species, with *Pseudois nayaur* constituting a genetically unique cluster closely associated with other *Caprinae* members.

Table 2.7 Genetic differentiation among Bharal and other species.

Species	*P. n. WII 01*	*p.n. nayaur*	*P. n. szechuanensis*	*Capra_hircus*	*Ovis aries*	*Boselaphus*	*Bubal_bubalis*	*Bos_gaurus*	*Ovibos_moschatus*	*Capricornis_thar_ jamrachi*	*Ovis_orientalis*	*B. taxicolor*	*Capra _ibex*	*Sus _scrof a*
P. n. WII 01														
P. n. nayaur	0.02													
P. n. szechuanensis	0.04	0.02												
Capra hircus	0.09	0.09	0.09											
Ovis_aries	0.1	0.1	0.1	0.1										
Boselaphus	0.14	0.13	0.13	0.1 3	0.14									
Bubal bubalis	0.14	0.14	0.14	0.1 4	0.14	0.13								
Bos_gaurus	0.14	0.14	0.14	0.1 4	0.14	0.13	0.12							
Ovibos_moschatus	0.11	0.11	0.11	0.1 1	0.11	0.13	0.14	0.14						
Capricornis thar_ jamrachi	0.11	0.11	0.11	0.1 1	0.11	0.14	0.14	0.14	0.08					
Ovis_ orientalis	0.1	0.1	0.1	0.1	0.2	0.14	0.14	0.14	0.11	0.11				
B. taxicolor	0.1	0.1	0.1	0.1	0.11	0.14	0.1 4	0.14	0.11	0.11	0.1 1			
Capra_ibex	0.09	0.09	0.09	0.03	0. 1	0. 14	0.14	0.14	0.11	0.11	0.1	0.1		
Sus_scrofa	0.19	0.19	0.19	0.1 9	0. 19	0. 19	0.1 9	0. 2	0.19	0.2	0.1 9	0.2	0. 19	

- **Ribosomal RNA and Transfer RNA genes**

The complete mitogenome of the Bharal has 22 tRNA genes and two ribosomal RNA genes. The 12S rRNA measured 957 bp, while the 16S rRNA measured 1573 bp (Table 2.4 and Fig. 2.6). Two rRNA sequences exhibited the following nucleotide percentages: A (36.9%), T (23.4%), C (22.1%), and G (17.7%) (table 2.8). The two rRNA genes of Bharal measured 2531 base pairs, constituting 15.01% of the whole mitogenome. The two rRNA had a total AT content of 61.30%, with AT and GC skew values of 0.022 and 0.110, respectively. The 22 tRNA genes were distributed throughout the whole mitogenome, with sizes varying from 60 base pairs (tRNAser) to 75 base pairs (tRNALeu). Among the 22 tRNA genes, eight were located on the L-strand and 14 on the H-strand (Fig. 2.3 and Table 2.5). The nucleotide makeup of 22 tRNA consisted of A (32.9%), T (31.0%), C (17.0%), and G (19.0%), with a total size of 1512 bp. tRNA exhibited an AT bias, with total AT and GC content of 63.9% and 36.0%, respectively. Positive skew values were observed for the AT content (0.029) and the GC content (0.055) (table 2.7). Table 2.2 enumerates the anti-codons of 22 tRNAs derived from Bharal. With the exception of tRNA ser, it failed to form a stable configuration with its dihydrouridine arm, while all 21 tRNA genes exhibited a shared secondary cloverleaf structure (Fig. 2.6).

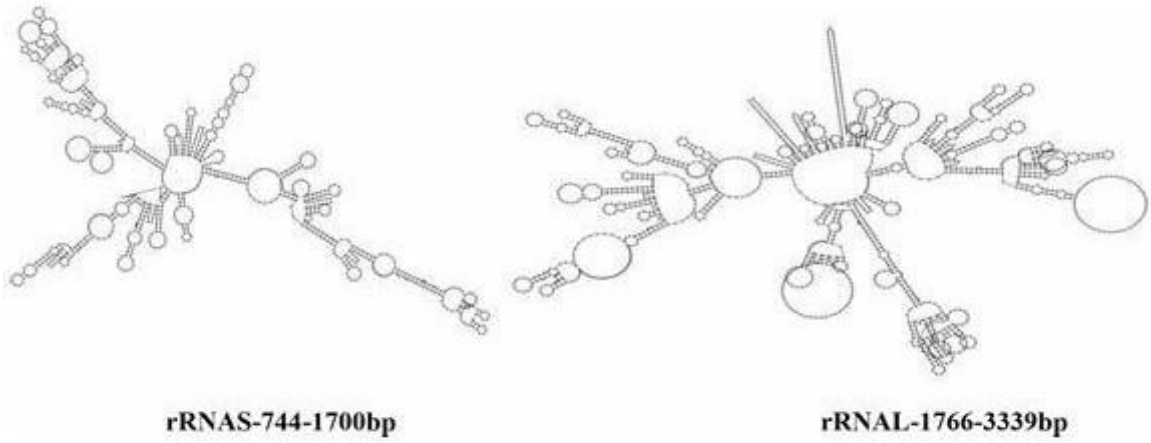

Figure 2.6. Predicted structures of the rRNA genes in *Pseudois nayaur*.

Table 2.8: Nucleotide composition and skewness in the *P. nayaur* mitochondrial genome							
Region	**Size (bp)**	**A (%)**	**T (%)**	**AT-skew**	**G (%)**	**C (%)**	**GC-skew**
Whole Mitogenome	16,718	33.6	26.3	0.121	13.2	26.9	0.341
PCGs	11,319	31.4	27.7	0.062	13	27.9	0.364
tRNAs	1,512	32.9	31	0.029	19	17	0.055
rRNAs	2,531	36.9	23.4	0.022	17.7	22.1	0.11
Control Region	1,285	37.8	24.2	0.211	12.3	26	0.436

- **Complete Mitogenome Phylogeny of *Pseudois nayaur***

A Bayesian inference phylogenetic study utilizing full mitochondrial genome sequences was conducted to assess the evolutionary connections of *Pseudois nayaur* (bharal) from India and China in relation to other bovid species. The resultant phylogenetic tree (Fig. 2.7) exhibited robust statistical support for the majority of clades (posterior probabilities > 0.97), signifying substantial confidence in the deduced connections. *P. nayaur* constituted a unique and well supported monophyletic clade within the *Caprini* tribe. Two principal lineages were identified within this group—one consisting of sequences from India (*P. nayaur*_India_01, indicated in red) and the other from China (indicated in blue). This distinct regional difference indicates a possible phylogeographic divergence and genetic structure within the species.

The western Himalaya (India) sequences grouped independently, whereas the Chinese sequences constituted a closely connected sub-clade, signifying regional difference. The *Pseudois* clade exhibited a tight evolutionary relationship with other *Caprini* members, including *Capra hircus, Capra ibex*, and several *Ovis* species, in accordance with prior classifications. The extensive tree topology classified *Caprini* as a sister group to *Boselaphini* (*Boselaphus*) and *Bovini* (*Bos gaurus, Bubalus bubalis*), with *Sus scrofa* serving as the outgroup for tree rooting. The tree topology underscores the unique evolutionary history of *P. nayaur* and emphasizes its phylogenetic diversity throughout its geographical distribution.

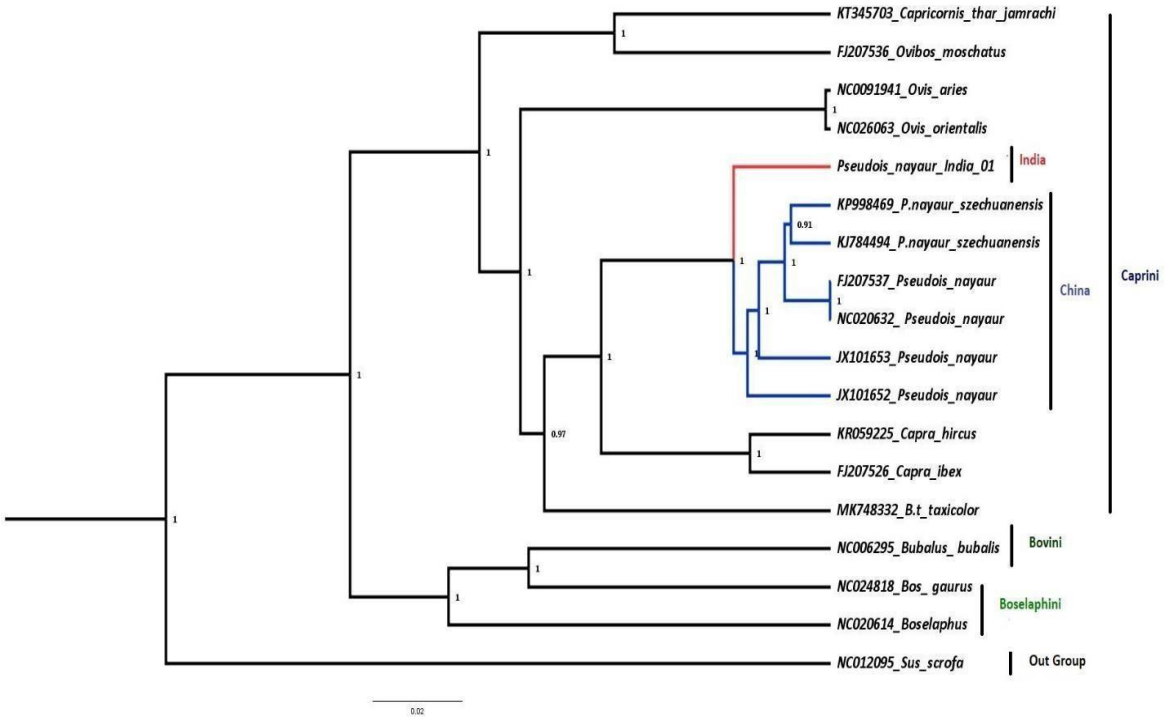

Figure 2.7. Phylogenetic relationship of *Pseudois nayaur* with other species of Bharal based on Complete mitogenome. Bayesian posterior probability values are shown at each node.

Divergence Dating and Evolutionary Genetics

Molecular dating study was performed using BEAST software (version 1.7) to determine the divergence periods among these lineages and other related species. A rigorous molecular clock model was utilized, supposing a uniform rate of mitochondrial DNA evolution among lineages. The molecular clock was calibrated utilizing fossil-derived temporal constraints. Two fossil calibration points were established: the divergence between *Bovidae* and *Moschidae* was approximated at 18 million years ago (Mya), while the separation between *Cervidae* and *Bovidae+Moschidae* was estimated at 17.2 Mya. These temporal markers functioned as dependable references to establish the evolutionary chronology. Sixteen full mitochondrial genomes from diverse species within the families *Cervidae, Bovidae*, and *Moschidae* were analyzed to enhance the evolutionary context and refine divergence estimations.

Bayesian inference utilizing Markov Chain Monte Carlo (MCMC) simulations was conducted, executing two separate studies of 10 million generations each, with sampling every 1,000 generations and discarding the initial 10% as burn-in. This

method produced a maximum clade credibility (MCC) tree, encapsulating the most probable phylogenetic connections and divergence periods among the species analyzed. The conclusive phylogenetic tree, shown with FigTree software, demonstrated a large divergence between the Indian and Chinese bharal lineages, corroborating the existence of past biogeographic separation. The phylogenetic placement of *P. nayaur* within the *Caprini* tribe was well corroborated, with its nearest evolutionary relatives being animals from the genera *Ovis* and *Capra*. These findings underscore the genetic uniqueness and evolutionary background of bharal, emphasizing the necessity of acknowledging regional lineages for biodiversity conservation and management approaches.

2.4 Results

- **Median-Joining Network Reveals Genetic Structure and Phylogeographic Patterns in *Pseudois nayaur***

  The median-joining (MJ) haplotype network, developed from mitochondrial control region sequences, offered more understanding of the genetic architecture and distribution of haplotypes within *Pseudois nayaur* species (Fig. 2.8). Thirty different haplotypes were discovered among the 47 examined sequences. The western Himalayan area exhibited a complicated and intricate network structure, indicating a significant level of haplotype diversity and interconnection among indiviual from Uttarakhand, Himachal Pradesh, and Ladakh. Central haplotypes, including H_1, H_2, and H_3, were disseminated throughout many populations, perhaps signifying ancestral or extensively spread lineages. This network structure facilitates the manifestation of past gene flow and common ancestry in the western Himalayas. The inhabitants of the eastern Himalayan and China Mountains constituted a closer and more isolated cluster, exhibiting minor linkage of haplotypes with those from the western area. The majority of Eastern haplotypes, especially those from Sikkim and China, exhibited tight linkage yet remained unique, separated from western haplotypes by several mutational steps. These findings corroborate the phylogenetic analysis results, highlighting the existence of limited gene flow and discrete genetic lineages across geographically isolated populations. The MJ network collectively emphasizes the phylogeographic differentiation of *Pseudois nayaur*, bearing substantial significance for comprehending evolutionary history and guiding conservation efforts.

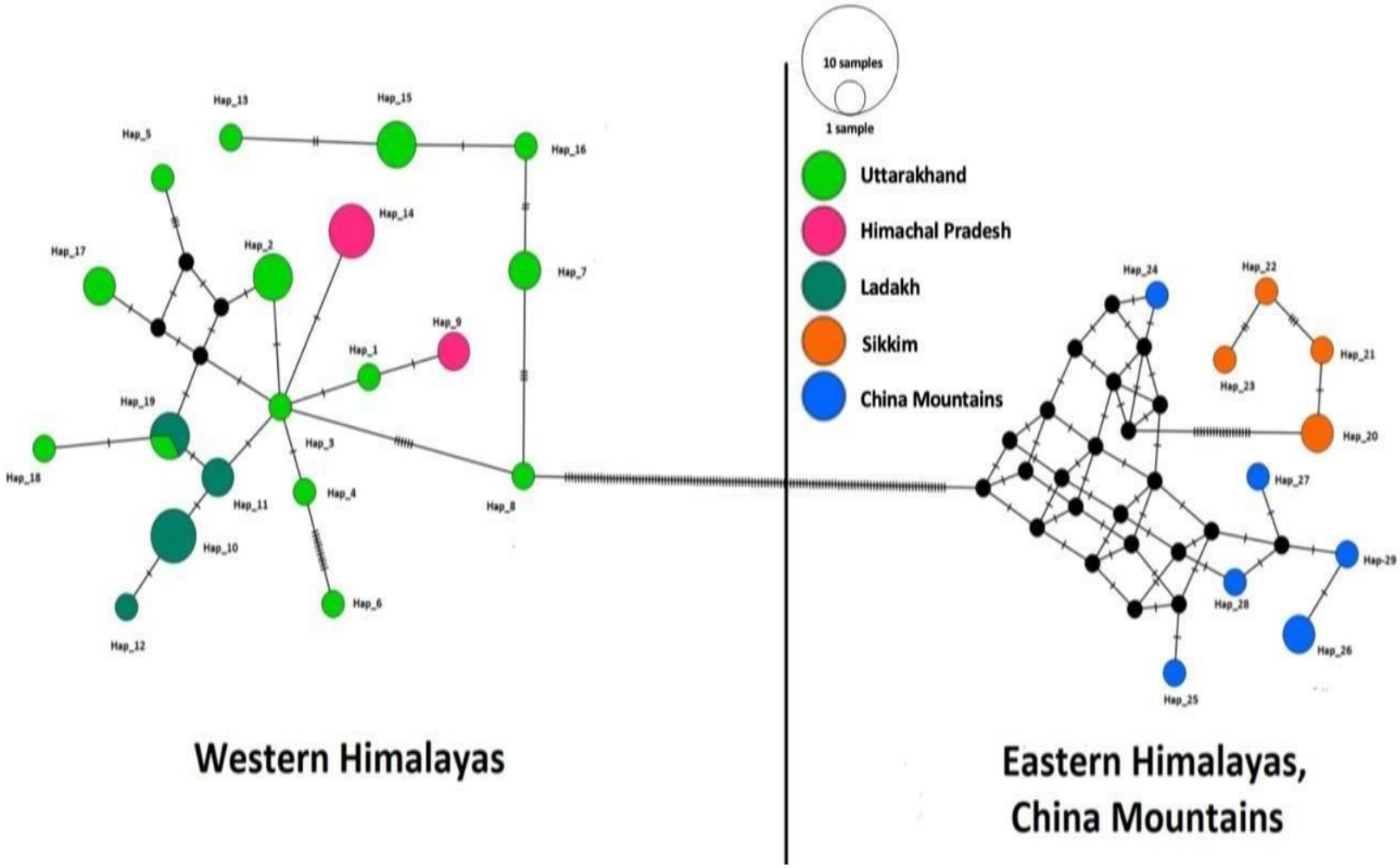

Figure 2.8. Median Joining Network Control region of Bharal sequence-based haplotype network, where different colours represent different populations. A sequence of lines shows the number of mutational stages

- **Phylogeographical Analysis of bharal**

A phylogenetic analysis was performed to examine the evolutionary connections across *Pseudois nayaur* populations, utilizing 47 mitochondrial control region sequences and one outgroup sequence (*Hemitragus jemlahicus*; NC_020628). Overall, 30 haplotypes with one out group. The Bayesian inference tree topology indicated the emergence of two separate and well-supported clades, with posterior probability values of 1.0, implying strong evolutionary diversification. The first clade comprised haplotypes HAP_01 to HAP_19, which predominantly represented individuals from the Western Himalayas, including Uttarakhand, Himachal Pradesh, and Ladakh. These series showed notable genetic homogeneity, suggesting a somewhat consistent genetic arrangement in this region. Comprising haplotypes HAP_20 to HAP_23, the second clade reflected eastern Himalayan population and mostly came from Sikkim. These haplotypes, which were far to the western Himalayas population, showed genetic uniqueness that suggested probable topographical or ecological restrictions influencing genetic difference. Comprising haplotypes HAP_24 to HAP_30, the third clade consisted only of Chinese Bharal samples. This clade showed notable genetic difference from Indian populations, and haplotype HAP_24, found at the base of the clade, would indicate an ancestral branch among the Chinese people. The phylogenetic structure demonstrated distinct regional divergence, indicative of ancient biogeographic processes and restricted gene flow across populations in the trans-Himalayan area (Fig. 2.9).

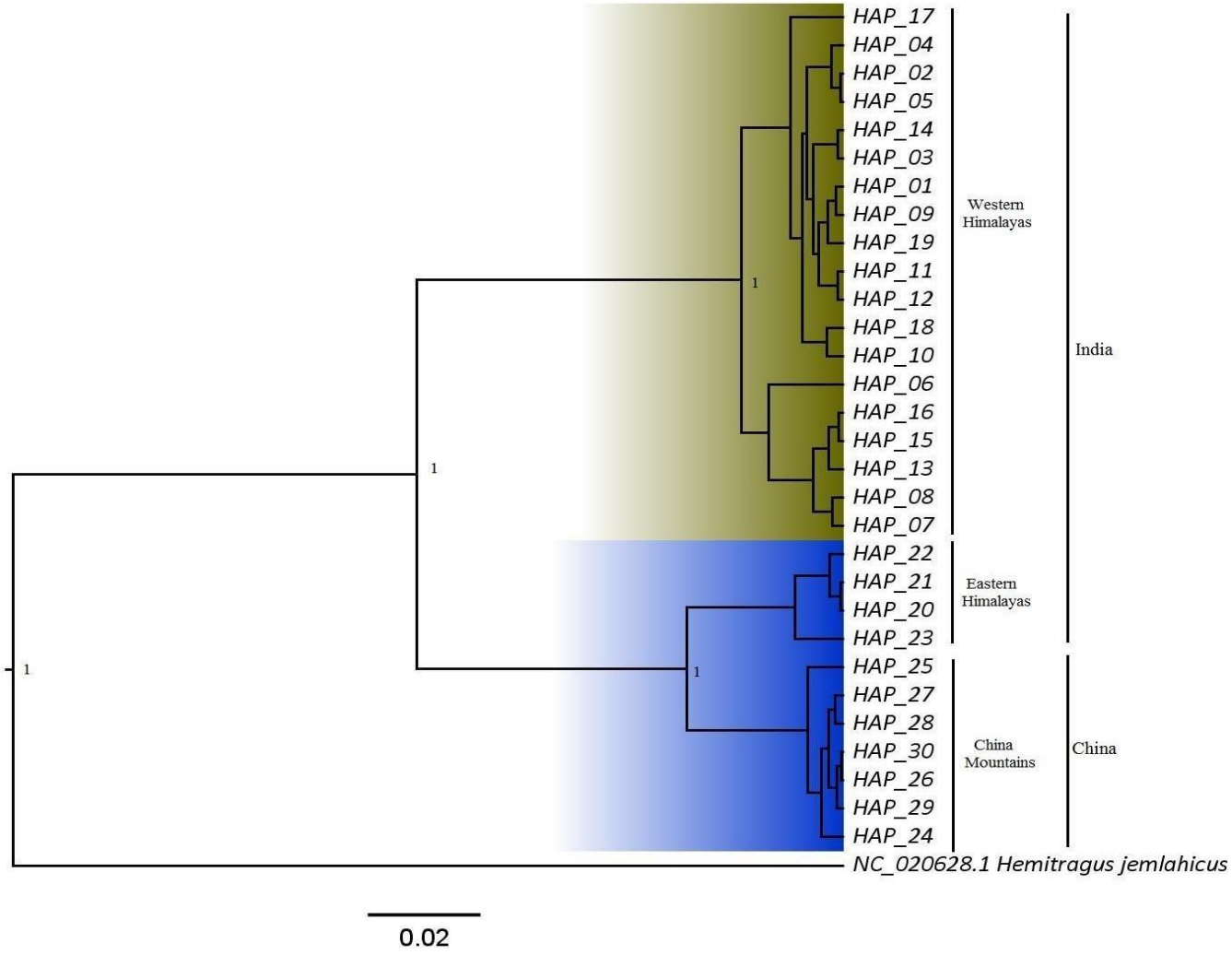

Figure 2.9. Phylogenetic relationship of Bharal based on control region with Bayesian posterior probability values at each node.

The phylogenetic tree (Fig. 2.10), constructed from mitochondrial *cytochrome b* (Cyt *b*) sequences, corroborates the control region results, illustrating the evolutionary relationships among *Pseudois nayaur* (Bharal) haplotypes from the western Himalayas, eastern Himalayas, and Chinese mountain ranges, with *Hemitragus jemlahicus* serving as the outgroup. The tree clearly separates the populations into two strongly supported clades (posterior probability = 1): a western Himalayan clade (Pamir Plateau) and an eastern Himalayan–Chinese clade (Tibetan Plateau and China Mountains), suggesting deep genetic divergence shaped by geographical barriers.

The western Himalayan clade (highlighted in yellow) includes haplotypes HAP_01–HAP_22, displaying marked internal structuring and shared ancestry, particularly among individuals from the Pamir Plateau and adjacent regions. The eastern Himalayan–Chinese clade (highlighted in blue) comprises haplotypes HAP_23–HAP_38, which further segregate into geographically coherent subclusters, including the Tibetan Plateau, Helan Mountains, and Qilian Mountains. Posterior probability values within this clade range from 0.7 to 1.0, indicating moderate to high confidence in the inferred branching patterns.

The basal placement of *H. jemlahicus* confirms its suitability as an outgroup and provides directionality for the observed divergences. Overall, the phylogeny reveals strong phylogeographic structuring in *P. nayaur*, with limited gene flow between western and eastern lineages and pronounced regional differentiation—patterns likely driven by historical climatic fluctuations and the formidable topographical barriers of the Himalayas and surrounding mountain systems.

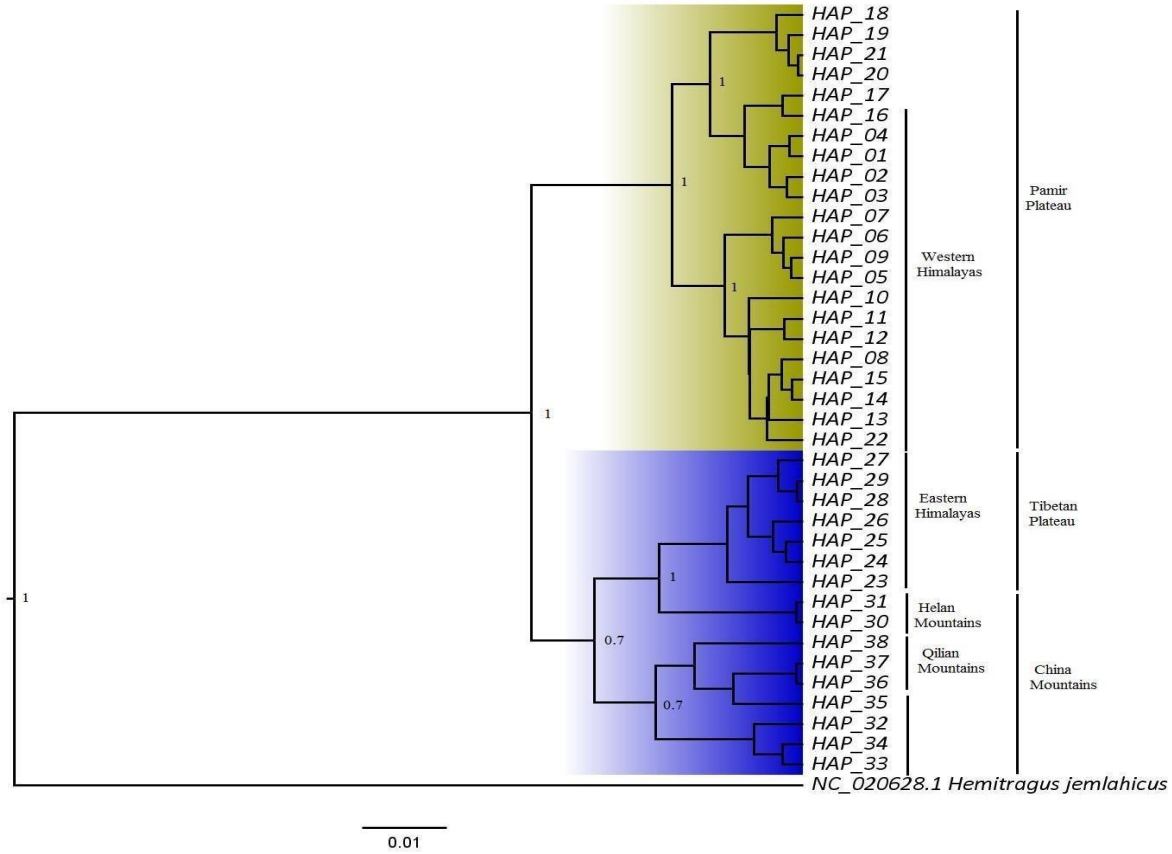

Figure 2. 10. Phylogenetic relationship of Bharal based on cyt *b* with Bayesian posterior probability values at each node.

- **Estimating Genetic Divergences**

The evolutionary history and genetic divergence of *Pseudois nayaur* (Bharal) are estimated using a time-calibrated maximum credibility tree based on the Cytocrome *b* (Cyt *b*) gene, as shown in figure 2.11. The x-axis of the tree denotes time in millions of years (Ma), spanning from the Miocene period through the Pliocene and into the Pleistocene. The tree includes both Himalayan Bharal (*Pseudois nayaur nayaur*, shown in red) and Chinese Bharal (*Pseudois nayaur szechuanensis*, marked in blue), with *Oryx gazella* functioning as an outgroup to establish the phylogeny's root. The divergence between *Pseudois nayaur* and *Oryx gazella* is believed to have transpired about 11.19 million years ago (Ma), during the mid-Miocene, signifying the basal break in the phylogenetic tree. Within *Pseudois nayaur*, two principal clades are discernible: one denoting the Himalayan Bharal and the other signifying the Chinese Bharal. The two subspecies diverged from a common ancestor roughly 1.38 million years ago, implying that physical and ecological factors in the Himalayan and trans-Himalayan regions drove their evolutionary separation during the Pleistocene period.

The Himalayan clade includes many haplotypes, specifically HAP_01 to HAP_17, that are closely related, indicating recent divergence and increased genetic closeness. For internal nodes within this clade, posterior probability values (e.g., 0.57, 0.80, 0.49) show modest to high support for certain lineage divergues. Mostly consisting of sequences taken from GenBank (e.g., JN839986.1, JN839985.1), the Chinese Bharal clade establishes a discrete group with internal divergence validated by posterior values of 0.61 and 0.78. The tree structure and divergence times suggest that both lineages have a quite recent common origin; geographic isolation and environmental variation most certainly affect their current genetic difference. This divergence study emphasizes the evolutionary distinctiveness of Himalayan Bharal and Chinese Bharal, therefore influencing taxonomy classification, conservation focus, and understanding of biogeographic patterns in the Asian highlands.

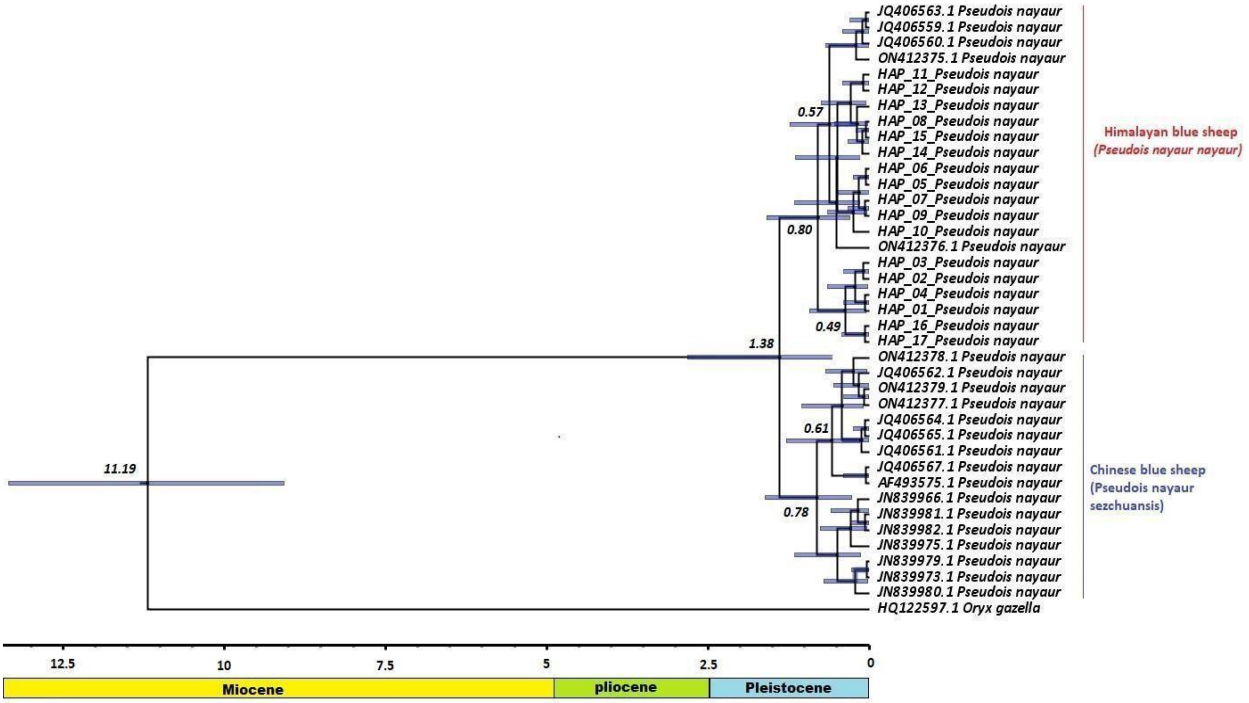

Figure 2.11. Divergent dating based on a cyt b gene maximum credibility tree. The x-axis shows time in million years.

2.5 Discussion

This study presents phylogeographic tendencies in *Pseudois nayaur* (Bharal), a representative Himalayan ungulate. Our findings significantly enhance the comprehension of the evolutionary history and biogeographic diversification of this species across the intricate Himalayan and trans-Himalayan terrains by integrating

comprehensive mitogenomic data, population-level sequence analyses, and sophisticated phylogenetic and species delimitation methodologies.

The median-joining (MJ) haplotype network developed from mitochondrial control region sequences provided significant insight into the genetic architecture and haplotype distribution within *Pseudois nayaur*. Thirty different haplotypes were discovered among 47 examined sequences. The western Himalayan region displayed a complex and reticulated network structure, indicating substantial haplotype diversity and connectivity among individuals from Uttarakhand, Himachal Pradesh, and Ladakh. Central haplotypes, such as H_1, H_2, and H_3, were shared across populations, suggesting ancestral or widely distributed lineages. In contrast, haplotypes from the eastern Himalayas (Sikkim) and China Mountains were more distinct and clustered, with minimal linkage to the Western region, highlighting the restricted gene flow and deep phylogeographic structuring across the range (fig 2.8). These patterns align with previous findings in Himalayan ungulates, such as *Pantholops hodgsonii* and *Naemorhedus goral*, where orographic barriers and niche differentiation contributed to east-west population structure (Chen et al., 2019; Groves & Grubb, 2011) (fig 2.8).

Phylogenetic analysis using mitochondrial control region sequences reinforced these patterns. The Bayesian inference tree revealed three distinct and well-supported clades: (1) western Himalayas, (2) eastern Himalayas, and (3) Chinese populations. The Chinese clades and eastern Himalayas, showed a relatively homogenous genetic structure, while the western Himalayas clades and Chinese clades exhibited clear separation, with HAP_24 positioned basally in the Chinese clade, indicating its ancestral nature. The discrete nature of these clades is indicative of long-term geographic isolation, supporting regional divergence driven by historical biogeographic processes (Fig 2.9). This structure mirrors phylogeographic separations observed in *Capra sibirica* and *Pseudois schaeferi* (Hassanin & Douzery, 2003) (Fig 2.9).

Similarly, phylogenetic reconstruction using Cyt *b* sequences yielded congruent results. Populations were grouped into two major clades: one corresponding to the western Himalayas (Pamir Plateau) and the other to the eastern Himalayas and Chinese regions (Tibetan Plateau and adjacent mountain systems). The western clade, containing haplotypes HAP_01 to HAP_22, showed internal structure and

signs of shared ancestry, while the eastern clade encompassed haplotypes HAP_23 to HAP_38 with greater substructuring across the Helan, Qilian, and Tibetan regions. Posterior probability values ranging from 0.7 to 1.0 lent moderate to high support to the inferred relationships. This deep geographic partitioning, supported by both control region and Cyt *b* data, underscores the effect of past topographic and climatic barriers on gene flow and lineage diversification.

Divergence dating based on Cyt *b* sequences revealed that *Pseudois nayaur* and *Oryx gazella* diverged approximately 11.19 million years ago during the Miocene. Within Pseudois nayaur, divergence between *P. n. nayaur* (Himalayan Bharal) and *P. n. szechuanensis* (Chinese Bharal) occurred around 1.38 million years ago, during the mid-Pleistocene—a period marked by significant climatic oscillations that likely drove habitat fragmentation and speciation (fig.2.11). This divergence timeframe is consistent with those recorded for other Himalayan mammals like *Procapra picticaudata* and *Moschus chrysogaster* (Jabin et al., 2023), reinforcing the role of Pleistocene glaciations and refugia in promoting allopatric divergence. Our findings also support the presence of cryptic sub-lineages within *P. nayaur*, consistent with Kim et al. (2015), who demonstrated that China's mountainous terrain can harbor hidden ungulate diversity(Fig. 2.11).

The complete mitogenome of *P. nayaur* (GenBank accession no. OP583799), spanning 16,718 bp, retained the standard vertebrate mitochondrial architecture with 13 protein-coding genes, 22 tRNAs, 2 rRNAs, and a control region (fig 2.3). This organization is conserved among Caprinae, as previously observed in *Ovis aries* and *Capra hircus* (Dou et al., 2016), reflecting strong evolutionary constraints on mitochondrial genome structure. The mitogenome showed a marked AT bias (~60%) and strand- specific nucleotide skews (positive AT-skew, negative GC-skew), with PCGs exhibiting average AT-skew of 0.062 and GC-skew of –0.364. These patterns are consistent with other high-altitude bovids like the yak (*Bos grunniens*) and Himalayan tahr (*Hemitragus jemlahicus*), and are likely shaped by strand- asymmetric replication and mutational pressures, potentially influencing codon selection and transcription efficiency(fig 2.3).

Codon usage analysis revealed a bias toward A- or T-ending codons, especially favoring leucine, isoleucine, and serine, as observed in other ruminants (Yang et al.,

2013). This reflects not only mutational preferences but also selective constraints for translational optimization under high-altitude adaptation. Intergenic overlaps, such as the 40 bp between ATP8 and ATP6, and the presence of incomplete stop codons (e.g., "T–" or "TA–") suggest space-efficient transcription, with reliance on post-transcriptional polyadenylation for translation completion. Similar observations were reported in *Capra aegagrus* and *Ovis ammon* (Mukai et al., 2017). tRNA structural analysis showed that all but tRNA-Ser (AGN) maintained the classical cloverleaf structure. The missing DHU arm in tRNA-Ser (AGN), a common feature across Caprinae and Cervidae (Dou et al., 2016), likely reflects relaxed functional constraints or evolved compensatory mechanisms in mitochondrial translation.

According to the complete mitogenome, pairwise comparisons within the Pseudois clade revealed low genetic distances (2–4%) *among P. n. nayaur, P. n. szechuanensis, and P. nayaur* WII_01, suggesting recent divergence. In contrast, deeper divergence (~0.09–0.10) was observed between *Capra hircus* and *Ovis aries*, consistent with their status as sister taxa within Caprinae (Ropiquet & Hassanin, 2006). Sus scrofa demonstrated the highest divergence (~0.20), validating its use as an outgroup and reflecting significant evolutionary distance, as also confirmed in broader artiodactyl phylogenies (Meredith et al., 2011).

The phylogenetic trees produced by both Bayesian approaches robustly endorsed the classification of *P. nayaur* into two major, well-delineated clades aligned with specific geographic locations. Haplotype network analysis corroborated these clades, demonstrating restricted haplotype sharing and increased mutational divergence in the eastern Himalayan compared with the western Himalayan clade, followed by a lower genetic distance between eastern Himalayan and Chinese populations. This structure reflects past biogeographic isolation, likely driven by the Himalayan orogeny and Pleistocene glacialinterglacial cycles that shaped regional distributions and genetic structures (Vintsek et al., 2022; Rana et al., 2023).

The results indicate considerable genetic diversity within the Bharal population throughout the two locations, accompanied by rather stable genetic characteristics. This combination of deep divergence, regional lineage structuring, and recent

diversification sheds light on the evolutionary dynamics of *Pseudois nayaur*, providing vital knowledge for future conservation planning and management.

Chapter-3

To assess the genetic diversity among the bharal population using mtDNA and microsatellites markers

3.1 Background

Genetic diversity is a cornerstone of species survival and adaptability, particularly for populations inhabiting ecologically fragile and topographically complex regions such as the Himalayas. The Bharal is a high-altitude caprine species distributed across Central Asia's mountain ranges, including India, Nepal, Bhutan, and China. In India, major populations occur in the trans-Himalayan regions of Ladakh, Himachal Pradesh, Uttarakhand, and parts of Sikkim. As a key prey species for apex predators such as the snow leopard (*Panthera uncia*), Bharal plays a pivotal role in maintaining the ecological balance of alpine and subalpine ecosystems.

Despite its ecological significance and broad geographic range, the genetic composition of bharal populations remains poorly documented. Emerging threats such as habitat fragmentation from infrastructure development, overgrazing by domestic livestock, climate change, and human–wildlife conflict may contribute to genetic isolation, reduced gene flow, and potential population bottlenecks. These factors highlight the urgency of assessing bharal genetic diversity and population structure to support long-term species persistence.

Molecular genetic tools offer powerful approaches for examining genetic variation, connectivity, and population structure. Mitochondrial DNA (mtDNA) markers particularly the control region and cytochrome *b* gene—are widely used due to the high mutation rate of the control region and the maternal inheritance of mtDNA, providing valuable insights into evolutionary history, population demography, and phylogeographic patterns. Nuclear microsatellite markers, known for their high polymorphism and codominant inheritance, are especially effective in detecting recent population structure, levels of inbreeding, and fine-scale genetic differentiation.

This study employs a dual-marker approach, combining mtDNA and microsatellite data to comprehensively assess genetic variation within and among bharal populations across the Indian Himalayas. This integrative analysis will enable the identification of Evolutionarily Significant Units (ESUs), Management Units (MUs), and regions most at risk of genetic erosion, based on both historical and contemporary processes. The findings will have direct implications for evaluating habitat connectivity, guiding conservation planning, and informing management strategies to ensure the long-term survival of bharal in the Himalayan landscape.

3.2 Methodology

- **Sample Collection and DNA Extraction**

This study involved the collection of 47 fecal samples of *Pseudois nayaur* from various parts of the Indian Himalayas for genetic analysis (table 3.1). Among them, 31 samples were collected from the Western Himalayas (WH), encompassing the Union Territory of Ladakh, Uttarakhand, and Himachal Pradesh, whereas 16 samples were collected from the Eastern Himalayas (EH), particularly from the state of Sikkim (Fig 3.1).

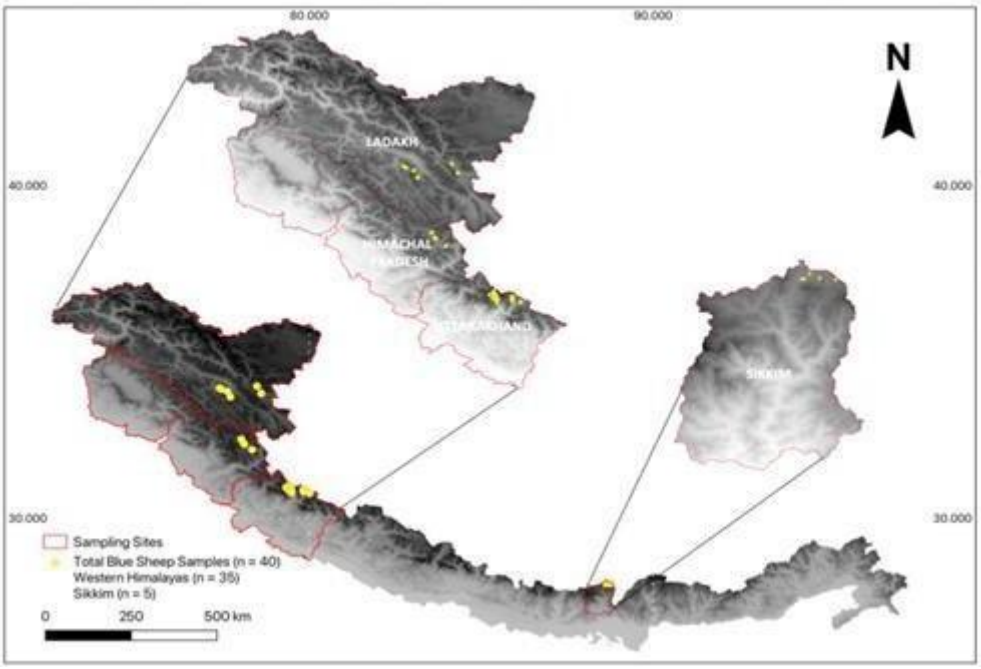

Fig. 3.1 A map showing the distribution and sampling locations for the current study. Yellow circles indicate the location of the sampling sites

Genomic DNA (gDNA) was isolated from fecal samples. For deteriorated samples (feces and bones), we employed a Gu-HCl-based silica binding technique, whereas DNA from fresh tissue samples was extracted utilizing the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The purity of DNA was assessed using 0.8% agarose gel electrophoresis stained with ethidium bromide, and its concentration was quantified using a QIAxpert spectrophotometer (Qiagen, Germany). All extractions were conducted in a specialized laboratory with rigorous contamination control measures. Approval from the Animal Ethics Committee was unnecessary as the samples were gathered non-invasively from feces or naturally died animals. The authors affirm that all experimental methods adhered to the appropriate laws and regulations.

Table 3.1: Sample details used for genetic analysis of Bharal from the Indian Himalayas

Region	Sample Type	n (Total)	Status
western Himalayas	Faecal, Tissue	31	Wild, Deceased
eastern Himalayas	Faecal	16	Wild

- **PCR Amplification & Cycle Sequencing**

Species identification and genetic diversity of *Pseudois nayaur* was conducted by sequencing of the mitochondrial cytochrome *b* gene and control region. PCR amplifications were conducted in 10 µL reaction volumes including 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 3 pmol of each primer, 0.6 units of DreamTaq DNA Polymerase (Thermo Fisher Scientific, USA), and 4 µL of genomic DNA template. The thermocycling protocol comprised an initial denaturation at 95 °C for 5 minutes, succeeded by 38 cycles of denaturation at 95 °C for 45 seconds, annealing at 56 °C for 45 seconds, and extension at 72 °C for 90 seconds, culminating in a final extension at 72 °C for 10 minutes. Positive and negative controls were incorporated to guarantee the reliability of amplification. PCR products were seen on a 2% agarose gel stained with ethidium bromide (0.5 µg/mL) and examined under a UV transilluminator.

Products that were successfully amplified underwent purification with alkaline phosphatase and Exonuclease I at 37 °C for 20 minutes, then followed by enzyme inactivation at 85 °C for 15 minutes. The purified amplicons were sequenced bidirectionally with the BigDye Terminator v3.1 Cycle Sequencing Kit and examined on an Applied Biosystems 3500 XL Genetic Analyzer.

- **Microsatellite Loci Amplification and Genotyping**

Eight microsatellite loci—INRA001 (Vaiman et al., 1994), Ca18 (Gaur et al., 2003), RT1 (Poetsch et al., 2001), FS59, FS120, SY58, SY112 (Chang et al 2012) and T156, (Jones et al., 2000) (Table 3.2) were chosen for the population genetic analysis of *Pseudois nayaur*. These loci were selected for their effective cross-amplification in related ungulate species. Multiplex primers were meticulously constructed according to molecular size and fluorescent dye labeling of the loci. In order to ensure accuracy and reduce amplification errors, each sample was subjected to multiplex PCR reactions in 10 μL volumes, comprising 5 μL of QIAGEN Multiplex PCR Buffer Mix (QIAGEN Inc.), 0.2 μM of each labeled forward primer (Applied Biosystems), 0.2 μM of each unlabeled reverse primer, and 20–100 ng of genomic DNA.

The PCR cycling protocol included an initial denaturation at 95 °C for 15 minutes, succeeded by 35 cycles including denaturation at 95 °C for 45 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, and a concluding extension at 60 °C for 30 minutes. Each batch had positive and negative controls to assess response fidelity. Amplified alleles were examined on an ABI 3500XL Genetic Analyzer (Applied Biosystems) with the LIZ 500 Size Standard (Applied Biosystems) and processed with GeneMarker version 2.7.4 (Hulce et al., 2011).

Table 3.2: Microsatellite Locus for Bharal (*Pseudois nayaur*) Population Genetic Analysis

LOCUS	**Primer Sequence**	**Size(bp)**	**dye**	**References**
FS59	F: GCGCGTATGTGTGTCTGAGT	261–273	FAM	Chang et al 2012
	R: TGTTGTTTTTAAGTCACCCAGTCT			
FS120	F: CAGTTTAGGGACTCTTCCTCTGG	237–255	PET	
	R: AACAGGATCCTTGCTGAACG			
SY58	F: CTATTGAACCTGTATCTCCCCC-3′	197–205	PET	Chang et al 2012
	R: GCATTCTGGCTCTGGCAA-3′			
SY112	F: TCA ATA ATC AGG GCA GGC TC-3´	192-202	6FAM	Chang et al 2012
	R: GTC CTT GTG TAG TCT GTG TGG G-3´			
T156	F: TCTTCCTGACCTGTGTCTTG	131-217	GREEN	Jones et al.,20 0
	R: GATGAATACCCAGTCTTGTCTG			
INRA	F: GGGTGTGACATTTTGTTCCC	110-170	FAM	Vaiman et al., 1994
	R:CTGCTCGCCACTAGTCCTTC			
CA18	F: TTCCGTCTCTCCCCTTAATA	128–136	FAM	Gaur et al., 2003
	R: TGGATCTGAGATTTCTGCTG			
RT1	F:TGCCTTCTTTCATCCAACAA	220-240	FAM	Poetsch et al., 2001
	R:CATCTTCCCATCCTCTTTAC			

Data Analysis

- **Mitochondrial DNA Sequence Analysis**

Sequences were acquired from both forward and backward orientations and subsequently aligned and modified with SEQUENCHER® v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequences were subsequently aligned individually utilizing CLUSTAL X v1.8 inside BioEdit (Hall, 1999). The Bayesian Information Criterion (BIC) was utilized for selecting the optimal DNA substitution models for phylogenetic tree reconstruction via MEGA 11. The TN93 + G + I model was utilized for the control region (CR) sequences, whereas the K2 + G model was employed for the *cytochrome b* (*cyt b*) sequences. Bayesian inference was performed with BEAST X v10.5 (Drummond et al., 2012) under a stringent molecular clock. The research was conducted over 10 million generations, sampling one tree every 10,000 generations, with a burn-in period of 5,000 generations. A 10% burn-in period was used, and parameter convergence was evaluated utilizing TRACER v1.7 (Drummond et al., 2012). The Bayesian Skyline Plot (BSP) was generated utilizing the MCMC approach in BEAST X v10.5, and genetic diversity indices, including haplotype diversity (Hd) and nucleotide diversity (π), were computed using DnaSP v5.10 (Librado and Rozas, 2009). Population history was assessed using Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) tests in conjunction with a mismatch distribution analysis.

- **Microsatellite Analysis**

Microsatellite genotyping was used on all genetically verified *Pseudois nayaur* samples. Of the eighteen loci examined, eight microsatellite markers were successfully amplified and applied for next research (Chang et al., 2012). The PCR amplification was carried out following the 2020 Gao et al. (2020) established technique. Using a Genetic Analyzer (Applied Biosystems, Foster City, USA), fluorescence-based genotyping was run with allele scoring done in GeneMapper v4.1 (Applied Biosystems, USA). Based on minimal or no genotyping errors, extremely tiny amplicon sizes, high amplification success rates, and strong discriminating ability to minimize overestimation of individuals, we chose eight loci for person identification. The count of distinct individuals was assessed utilizing GeneCap v1.2.2 (Wilberg et al., 2004), while the polymorphic information content (PIC) for

each locus was computed with CERVUS v3.0 (Kalinowski et al., 2007). Genetic diversity indicators, such as the number of observed alleles (Na), effective alleles (Ne), observed heterozygosity (Ho), anticipated heterozygosity (He), and Wright's inbreeding coefficient (FIS) for each locus, were computed utilizing GENALEX v6.5 (Peakall et al., 2012).

3.3 Results

- **Demographic Analysis**

The gene diversity analysis of the control region in several regions indicates diverse patterns of genetic variation (Table 3.3). The population in the western Himalayas demonstrates considerable haplotype diversity (Hd = 0.96) and nucleotide diversity (π = 0.0183), suggesting a rich genetic reservoir. In Ladakh, a reduced nucleotide diversity (π = 0.0069) is noted, accompanied by high haplotype diversity (Hd = 0.8), indicating high genetic variation. The population of Himachal Pradesh has the moderate haplotype diversity (Hd = 0.53) and nucleotide diversity (π = 0.0032), reflecting limited genetic variation within the region. In Uttarakhand, the population exhibits elevated haplotype diversity (Hd = 0.95) and nucleotide diversity (π = 0.0265), implying a high level of genetic variability. The eastern Himalayas show high haplotype diversity (Hd = 0.91) and nucleotide diversity (π = 0.0112), high genetic diversity. The total sample (n = 40) exhibits high haplotype diversity (Hd = 0.97) and moderate nucleotide diversity (π = 0.0609), suggesting overall genetic stability and diversity across the sampled regions.

Table 3.3. Gene diversity of control region (h), nucleotide diversity (π), for control region.

Location	Number of Samples	Number of Haplotypes (H)	Haplotype Diversity (Hd)	Nucleotide Diversity (π)
Western Himalayas	35	19	0.96	0.0183
Ladakh	8	4	0.8	0.0069
Himachal Pradesh	6	2	0.53	0.0032

Uttarakhand	21	13	0.95	0.0265
Eastern Himalayas	5	4	0.91	0.0112
Total	40	23	0.97	0.0609

Table 3.4 displays the overall gene diversity of the cytochrome *b* (Cyt *b*) region across several Himalayan regions, specifically the western Himalayas, eastern Himalayas. In the western Himalayas, six haplotypes were observed, with a haplotype diversity (Hd) of 0.7 and a nucleotide diversity (π) of 0.0053, indicating moderate genetic variation. The eastern Himalayas exhibited four haplotypes with high haplotype diversity (Hd = 0.99) and nucleotide diversity (π = 0.0261), reflecting a substantial level of genetic variability within the population. In the Indian Himalayas, 10 haplotypes were identified, with a haplotype diversity of 0.76 and a nucleotide diversity (π = 0.00994), suggesting moderate genetic differentiation across the region. Overall, the results indicate that gene diversity in the cyt *b* region is consistently distributed across these Himalayan regions, with no notable deviations suggesting population expansion or contraction.

Table 3.4. Gene diversity of cyt b (h), nucleotide diversity (π)

Location	**No. of Haplotype (H)**	**Haplotype Diversity (Hd)**	**Nucleotide Diversity (π)**
Western Himalayas	6	0.7	0.0053
Eastern Himalayas	4	0.99	0.0261
Overall	10	0.76	0.00994

The genetic diversity indices presented in Table 3.5 compare eight microsatellite loci across *P. n. nayaur* and *P. n. szechuanensis* populations, revealing moderate to high genetic variation in both groups. On average, *P. n. nayaur* exhibited slightly higher allelic richness (Na = 5.75) and effective number of alleles (Ne = 3.936) than *P. n. szechuanensis* (Na = 3.875; Ne = 2.887), indicating greater overall genetic variability. Observed heterozygosity (Ho) and expected heterozygosity (He) were also generally higher in *P. n. nayaur* (Ho = 0.632; He = 0.689) compared to *P. n. szechuanensis* (Ho = 0.626; He = 0.612), though both populations showed relatively balanced heterozygosity levels. Several loci (e.g., FS120 and CA18) in *P. n. nayaur*

demonstrated high polymorphism information content (PIC > 0.80), supporting their informativeness for population genetic studies. Most fixation index (F) values were negative, particularly in *P. n. szechuanensis*, suggesting an excess of heterozygotes and possible outbreeding or avoidance of inbreeding. The overall nuclear DNA result of Ho was 0.67and He was 0.65, followed by Na was 4.81. Shannon's Information Index (I) was 1.26 also showed the population is stable, followed by Fixation index (F) -0.12 showed the moderate genetic diversity.

The results indicate considerable genetic diversity within the Bharal population throughout the two locations, accompanied by rather stable genetic characteristics, suggesting evolutionary resilience and limited recent bottleneck effects. Collectively, the findings support the existence of healthy gene pools in both lineages, though slightly elevated genetic diversity in *P. n. nayaur* may reflect broader distribution, larger population size, or more extensive historical gene flow.

Table 3.5: Genetic Diversity Indices for Different Loci in Populations *P. n. nayaur* and *P. n. szechuanensis.*

Pop	Locus	N	Na	Ne	I	Ho	He	F	PIC
P. n. nayaur	**FS120**	35	9.000	7.292	2.080	0.514	0.863	0.875	0.881
	FS59	36	3.000	2.610	1.014	0.833	0.617	0.626	0.623
	INRA	36	6.000	3.939	1.481	0.500	0.746	0.757	0.803
	RT1	36	7.000	5.773	1.822	0.806	0.827	0.838	0.786
	SY112A	36	5.000	3.384	1.392	0.528	0.704	0.714	0.610
	SY58	35	4.000	1.641	0.726	0.200	0.391	-0.396	0.579
	CA18	36	6.000	4.386	1.613	0.889	0.772	-0.783	0.814
	T156	36	6.000	2.457	1.212	0.750	0.593	-0.601	0.632
P. n. szechuanensis	**FS120**	11	6.000	5.378	1.736	0.818	0.814	-0.853	0.788
	FS59	14	3.000	2.178	0.876	0.857	0.541	-0.561	0.453
	INRA	14	5.000	2.800	1.257	0.571	0.643	0.667	0.803
	RT1	12	4.000	3.470	1.317	0.833	0.712	0.743	0.663
	SY112A	14	4.000	1.712	0.832	0.357	0.416	-0.431	0.691
	SY58	14	2.000	1.912	0.670	0.500	0.477	-0.495	0.363
	CA18	14	4.000	2.992	1.177	0.643	0.666	-0.690	0.601
	T156	14	3.000	2.649	1.035	0.429	0.622	0.646	0.551
Mean		**24.563**	**4.813**	**3.411**	**1.265**	**0.677**	**0.650**	**-0.124**	**0.7257**
SE		**2.897**	**0.449**	**0.399**	**0.102**	**0.052**	**0.036**	**0.036**	**-**

Note: N= number of samples, Na = number of alleles, Ne = No. of Effective Alleles, I = Shannon's Information Index, *H*O = observed heterozygosity; *H*E = expected heterozygosity, F=fixation index, PIC = Polymorphic Information Content

3.4 Discussion

The genetic diversity analysis of Bharal populations in the Himalayan regions revealed pronounced patterns of variation, with distinct genetic structures evident in the western and eastern Himalayas. In the western Himalayas, nucleotide diversity ($\pi = 0.0183$) and haplotype diversity (Hd = 0.96) were notably high, suggesting a neutral evolutionary process and stable population dynamics. This pattern is consistent with Singh et al. (2023) for the Himalayan tahr (*Hemitragus jemlahicus*), which also reported high genetic diversity in the western Himalayas, and with Cancellare (2023) for snow leopard (*Panthera uncia*) populations in the same region, both studies reinforcing the inference of stable, neutral evolutionary processes.

Conversely, the Ladakh population exhibited lower nucleotide diversity ($\pi = 0.0069$) and moderate haplotype diversity (Hd = 0.8), indicative of possible population expansion or selective sweeps. This trend parallels Tumendemberel (2020) for the black bear in the eastern Himalayas, where selective pressures or declines were proposed (Table 3.5). Although no definitive bottleneck or demographic contraction was detected in our data, the genetic differentiation between Ladakh and western Himalayan populations suggests a complex evolutionary history shaped by environmental conditions and potential selection mechanisms (Table 3.5).

The Himachal Pradesh population displayed the lowest genetic variation, with haplotype diversity (Hd = 0.53) and nucleotide diversity ($\pi = 0.0032$). This contrasts with Jabin et al. (2023) for Himalayan ibex (*Capra sibirica*) in the eastern Himalayas, who attributed declining nucleotide diversity to habitat fragmentation and environmental stress. In contrast, our results indicate a relatively consistent population with neutral evolutionary tendencies and no clear evidence of substantial fragmentation or environmental stress in Himachal Pradesh.

In the eastern Himalayas, populations showed nucleotide diversity ($\pi = 0.0112$) and high haplotype diversity (Hd = 0.91). These values are broadly comparable to Sharief et al. (2025) for musk deer in the same region, where high haplotype diversity was observed alongside signs of population decline—signals that were not strongly evident in our study. Nevertheless, the observed patterns may still reflect the influence of localized environmental or ecological pressures on population genetic structure.

Across all sampled regions, mtDNA analysis revealed high haplotype diversity (Hd = 0.97) and moderate nucleotide diversity ($\pi = 0.0609$), indicating a stable and well-preserved population structure. This aligns with Dolker et al. (2023) for Bharal populations in Tibet, where high genetic diversity was attributed to the effects of population isolation on genetic variation. Nuclear DNA results showed observed heterozygosity (Ho) of 0.67, expected heterozygosity (He) of 0.65, and mean number of alleles (Na) of 4.81. The fixation index (F) was −0.12, indicating moderate genetic diversity without inbreeding, and Shannon's information index (I) of 1.26 supported the conclusion of population stability across the Himalayan range.

Chapter-4

To investigate population genetic structure, gene flow, and demographic history of bharal

4.1 Background

Bharal (*Pseudois nayaur*) are a keystone herbivore inhabiting the high-altitude landscapes of the Indian Himalayan Region (IHR). As a principal prey species for apex predators such as the snow leopard (*Panthera uncia*), they play a critical role in maintaining ecosystem balance (Schaller, 1977; Mishra et al., 2004). Their extensive morphological and ecological adaptations enable survival in some of the most extreme environments on Earth, allowing them to occur widely across the Himalayas, the Tibetan Plateau, and adjacent high-altitude ranges (Groves & Grubb, 2011; Wilson & Mittermeier, 2011). Despite this broad distribution, the genetic structure, gene flow, and demographic history of Bharal populations in the western Himalayas (WH) and eastern Himalayas (EH) remain poorly understood.

Molecular phylogenetic studies have substantially advanced our understanding of high-altitude ungulate diversity, including that of Bharal. Previous research has revealed marked genetic differentiation across populations spanning the Himalayas and Tibetan Plateau, indicating the presence of two distinct evolutionary lineages. Recent analyses suggest that EH populations are genetically closer to *P. n. szechuanensis*, the Chinese Bharal largely restricted to the Tibetan Plateau, whereas WH populations are more closely aligned with *P. n. nayaur*. This divergence is likely shaped by historical glacial cycles, river systems, complex topography, and other biogeographic barriers constraining gene flow and population connectivity (Avise, 2000; Hewitt, 2004).

While these broad phylogeographic patterns are increasingly evident, fine-scale studies addressing intra-population genetic variation and the role of environmental variables in shaping population structure remain scarce. The mechanisms underlying past connectivity between WH and EH populations, and the extent of contemporary gene flow, are also largely unexplored. Understanding these factors is essential for

conservation, particularly given the substantial genetic differentiation between the two regions (Erard et al., 2004).

To address these knowledge gaps, this study investigates gene flow, demographic history, and population genetic structure of Bharal across the IHR, alongside their evolutionary relationships inferred from mitochondrial DNA and microsatellite markers. We hypothesise that WH and EH populations represent distinct Evolutionarily Significant Units (ESUs), requiring region-specific conservation approaches. By clarifying patterns of genetic variation and phylogenetic relationships, this work aims to inform future management strategies for this ecologically important high-altitude ungulate.

4.2 Methodology

- **Sample Collection and DNA Extraction**

For this study, 47 fecal samples of *Pseudois nayaur* were collected from diverse locations in the Indian Himalayas for genetic analysis. While 16 samples came from the eastern Himalayas (EH), most famously from Sikkim (Fig 4.1), 31 samples were collected from the western Himalayas (WH), including the Union Territory of Ladakh, Uttarakhand, and Himachal Pradesh.

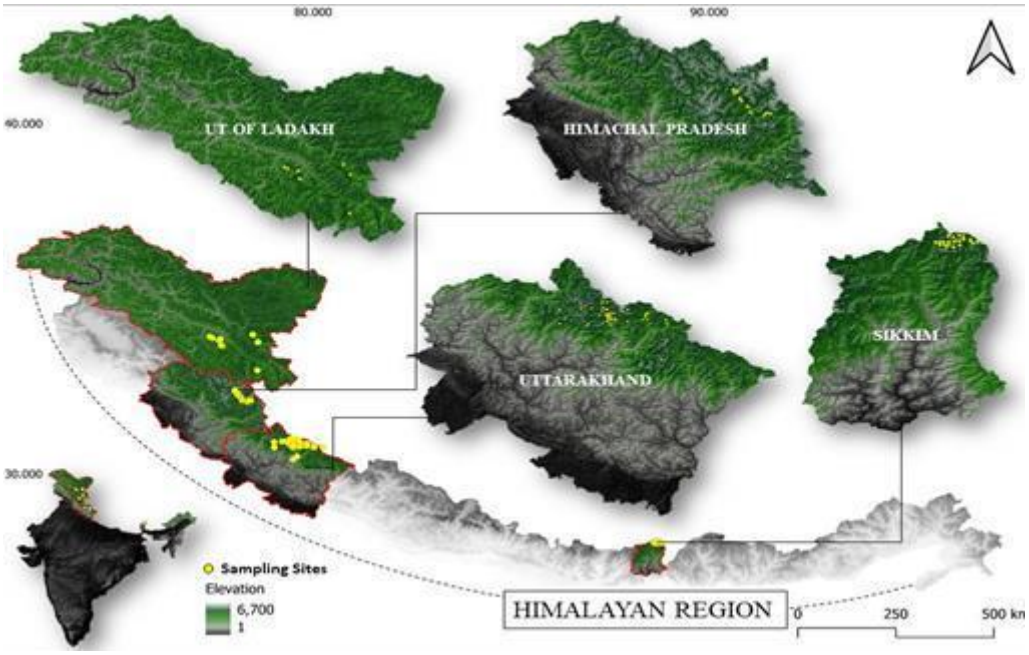

Fig. 4.1 A map showing the distribution and sampling locations for the current study. Yellow circles indicate the location of the sampling sites.

Genomic DNA (gDNA) was isolated from fecal specimens. For deteriorated samples (feces and bones), we employed a Gu-HCl-based silica binding technique, whereas DNA from fresh tissue samples was extracted utilizing the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The purity of DNA was assessed using 0.8% agarose gel electrophoresis stained with ethidium bromide, and its concentration was quantified using a QIAxpert spectrophotometer (Qiagen, Germany). All extractions were conducted in a specialized laboratory with rigorous contamination control measures. Approval from the Animal Ethics Committee was unnecessary as the samples were gathered non-invasively from feces or naturally died animals. The authors affirm that all experimental methods adhered to applicable rules and legislation.

- **PCR Amplification**

Polymerase chain reaction (PCR) amplifications were conducted in 20µl volumes including 10–20 ng of extracted genomic DNA, 1× PCR buffer (Applied Biosystems), 2.5mM MgCl2, 0.2 mM of each dNTP, 5 pmol of each primer, and 5 units of Taq DNA polymerase (Thermo Scientific). PCR amplification was performed using 23 overlapping segments of complete mitochondrial DNA (Hassanin et al., 2012). We also included fragments of the whole Cytochrome b (Bhatt et al., 2020) and Cytochrome c oxidase subunit-I gene (Kundu et al., 2019) to improve the overlap. The PCR process for DNA amplification consisted in an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 54–56°C for 40 years, extension at 72°C for 50 years, and a last extension at 72°C for 15 years. One assessed the accuracy and efficiency of the responses by use of controls. PCR results were electrophoresis on a 2% agarose gel under UV light and using Ethidium bromide dye. To eliminate any remaining primer, the amplified PCR products were subjected to 15 minutes at 37ºC and 80ºC respectively using Exonuclease I and Shrimp alkaline phosphatase (USB, Cleveland, OH). The pure amplicons were then sequenced bidirectionally using BigDye Terminator 3.1 and an automated Genetic Analyser 3500XL (Applied Biosystems, Carlsbad, CA, USA).

- **Microsatellite loci amplification and genotyping**

Eight microsatellite loci—INRA001 (Vaiman et al., 1994), Ca18 (Gaur et al., 2003), RT1,(Poetsch et al., 2001), FS59, FS120, SY58, SY112 (Chang et al 2012) and T156,

(Jones et al., 2000) were chosen for the population genetic analysis of *Pseudois nayaur*. These loci were selected for their effective cross-amplification in related ungulate species. Indiviualy of multiplex were meticulously constructed according to molecular size and fluorescent dye labeling of the loci.

To ensure accuracy and reduce amplification errors, each sample was subjected to multiplex

PCR reactions in 10 μL volumes, comprising 5 μL of QIAGEN Multiplex PCR Buffer Mix (QIAGEN Inc.), 0.2 μM of each labeled forward primer (Applied Biosystems), 0.2 μM of each unlabeled reverse primer, and 20–100 ng of genomic DNA. The PCR cycling protocol included an initial denaturation at 95 °C for 15 minutes, succeeded by 35 cycles including denaturation at 95 °C for 45 seconds, annealing at 55 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 60 °C for 30 minutes. Each batch had positive and negative controls to assess response fidelity. Amplified alleles were examined on an ABI 3500XL Genetic Analyzer (Applied Biosystems) with the LIZ 500 Size Standard (Applied Biosystems) and processed with GeneMarker version 2.7.4 (Hulce et al., 2011).

- **Data Analysis**

Sequences adopted from both forward and reverse orientations were aligned and altered using SEQUENCHER® v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Later, the sequences were individually aligned using CLUSTAL X v1.8 within BioEdit (Hall, 1999). MEGA 11 (Tamura et al., 2021) allowed one to calculate the genetic p-distance among sequences. Using MEGA 11, the best DNA substitution models were chosen using the Bayesian Information Criteria (BIC), for phylogenetic tree reconstruction. The control region (CR) sequences used the TN93 + G + I model; the cytochrome *b* (cyt *b*) sequences used the K2 + G model.

Using STRUCTURE 2.3.4 (Pritchard et al., 2000), we performed a Bayesian clustering analysis to determine the most likely count of genetic groups in India. Presuming allele frequencies to be independent, we investigated our data using the admixture model with a burn-in of 10,000 and then 100,000 MCMC (Markov chain Monte Carlo) replications. Ten separate experiments for every cluster configuration (K) ranging from 1 to 10 were carried out. Using the online program STRUCTURE HARVESTER (Earl 2012), we determined the statistically best number of clusters

(K) by using the ΔK measure. Using the ADEGENET program, the DAPC method was used to count the genetic clusters in the population (Jombart et al., 2010). Without requiring Hardy-Weinberg equilibrium, DAPC is a multivariate, model-free strategy that maximizes genetic difference across groups with unknown prior clusters thereby improving population discrimination. FCA was performed using the GENETIX 4.05 program (Belkhir 2004). CONVERT 1.31 (Glaubitz 2004) was utilized to transform the necessary input file format. Pairwise FST values among the populations were computed using GenAlEx version 6.5 (Peakall et al., 2006).

- **Predictor Selection for Suitability Modeling Using MaxEnt**

A dataset including 14 predictor factors was first employed to simulate the likely distribution of Bharal. We utilized six bioclimatic layers sourced from the Worldclim database (Fick and Hijmans 2017), four topographic variables (elevation, slope, aspect, and terrain ruggedness index) from the SRTM dataset (http://srtm.csi.cgiar.org/srtmdata), and two land use/land cover classes (Barren land and Grassland) from https://lpdaac.usgs.gov/products/mcd12q1v006/. We created distinct Euclidean layers for highways and rivers to examine if these factors affect the distribution of Bharal. Among the 14 variables, we analyzed their collinearity (Spearman correlation coefficient $r > 0.7$), yielding seven uncorrelated variables for the western Himalayas and six for the eastern Himalayas ($r < 0.7$). Only a limited number of factors were considered physiologically important for the ecological requirements of Bharal from both regions. They were employed for additional analysis after scaling the selected variables to 30 arc seconds, or approximately 1 km near the equator. Analysis was conducted using Arc GIS v10.5 and the R package 'coordinatecleaner' (Zizka et al. 2019). Model procedures were executed using MaxEnt v3.4.4 (Phillips & Dudík 2024) subsequent to the analysis of Bharal distribution and environmental variable data. The model's maximum iterations were established at 500, with 10 cycles designated, cross-validation selected for repetition classification, and the substantial impact of chosen environmental factors on predicting the suitable distribution area of Bharal assessed.

- **Spatial genetic analysis**

The correlation between paired genetic and geographic distances was analyzed to identify the pattern of isolation by distance among the separated regions, utilizing Mantel's test as implemented in Alleles in Space 1.0 (Miller et al., 2005).

4.3 Results

- **Pairwise Genetic Differentiation**

The pairwise genetic differentiation values (below the diagonal) and their corresponding standard errors (above the diagonal) among six mitochondrial genome sequences of *Pseudois nayaur* (Bharal), including different subspecies are shown in table 4.1. The greatest genetic difference (4.1%) is noted in *Mitochondrial_genome_India_1* and *JX101653.1_P. nayaur*, signifying considerable divergence. The minimal difference (1.9%) is seen between *NC_020632.1_P. nayaur* and both *KP998469.1_P. n. szechuanensis* and *KJ784494.1_P. n. szechuanensis*, indicating close genetic affiliations. The *P. n. szechuanensis* sequences exhibit minimal genetic divergence both among themselves and in comparison, to *NC_020632.1_P. nayaur*, whereas *JX101652.1_P. n. nayaur* has significant difference from all other sequences. The consistently low standard errors (between 0.001 and 0.002) signify a high level of confidence in the results. These findings underscore differing levels of genetic variability within populations, indicating potential historical isolation, geographic diversification, or subspecies-level organization among *Pseudois nayaur*.

Table 4.1. Genetic differentiation among Bharal species of *Pseudois nayaur* are presented (below the diagonal) and their standard errors (above the diagonal).

Species	1.	2.	3.	4.	5.	6.
Mitochondrial_genome_India_1	-	0.002	0.002	0.002	0.002	0.002
JX101653.1_P. nayaur	0.041	-	0.002	0.002	0.002	0.002
NC_020632.1_P. nayaur	0.038	0.033	-	0.001	0.001	0.002
KP998469.1_P.n. szechuanensis	0.039	0.035	0.019	-	0.001	0.002
KJ784494.1_P.n. szechuanensis	0.038	0.033	0.019	0.020	-	0.002

JX101652.1_P.n.nayaur	0.037	0.036	0.034	0.035	0.034	-

- **Demographic Analysis**

The outcomes of demographic analysis for several populations of Bharal (*Pseudois nayaur*) employing Tajima's D and Fu's Fs statistics (table 4.2). The *P. n. nayaur* population has a marginally positive Tajima's D (0.46) and a significantly negative Fu's Fs (-5.417), but non-significant P – value indicating potential historical stable selection rather than current population growth. Conversely, *P. n. szechuanensis* has positive values for both statistics (Tajima's D = 1.365; Fu's Fs = 0.461), but non-significant P – value suggesting population in equilibrium stage. Finally, The *P. nayaur* population has a negative Tajima's D (-0.875) and Fu's Fs (-0.63), over all Bharal population in equilibrium stage due to non-significant P – value of cyt *b*.. Collectively, these findings suggest that none of the groups demonstrate substantial evidence of recent expansion.

Table 4.2. Demographic analysis shows the Bharal populations had not undergone an expansion

Population	**Tajima's Da**	**Fu's Test (Fs)**	**P-value (Tajima'D and Fu's Test)**
P.n.nayaur	0.46	-5.417	P > 0.10
P.n.szechuanensis	1.365	0.461	P > 0.10
P.nayaur	-0.875	-0.63	P > 0.10

The neutrality test findings for the control region (D-loop) region across several geographic populations of *Pseudois nayaur* show no significant variation from neutrality, with Tajima's D and Fu's Fs values yielding P > 0.10. The western Himalayas, Ladakh, Himachal Pradesh, and Uttarakhand all had positive Tajima's D and Fu's Fs values, but non-significant P – value indicating potential historical stable. Although the eastern Himalayas had a negative Tajima's D (-0.6464), but non-significant P – value which would suggest a population is stable (table 4.3). Likewise,

the total population showed almost neutral values (D = -0.1243; Fs = 5.3), but non-significant P – value So supporting the inference that the Bharal populations over the Himalayan range are essentially developing under neutral conditions with strong evidence of demographic stability.

Table 4.3. Neutrality Test using Tajima's (D), and Fu's (Fs) for control region.

Location	Tajima's D	Fu's Test (Fs)	P-value (Tajima's D and Fu's Test)
Western Himalayas	0.3944	0.033	P > 0.10
Ladakh	1.4025	3.011	P > 0.10
Himachal Pradesh	1.2188	3.696	P > 0.10
Uttarakhand	1.0116	0.825	P > 0.10
Eastern Himalayas	-0.6464	1.467	P > 0.10
Total	-0.1243	5.3	P > 0.10

The Bayesian skyline map, shown in Figure 4.2 shows a comparatively constant effective population size across time. Demographic stability in the species' evolutionary history is suggested by the lack of clear signs of either population growth or decrease. The 95% greatest posterior density (HPD) intervals are shown by the shaded region, which shows some degree of uncertainty but a generally stable population size over time.Figure (b) illustrates the mismatch distribution study by comparing the actual pairwise differences (blue dashed line) with the predicted distribution under a unimodal model of rapid population increase (red line). The observed curve suggests that the population has not experienced recent demographic growth due to its multimodal form and deviation from the anticipated unimodal distribution. This is further supported by a difference between the actual and expected distributions. All of these findings point to a long history of demographic stability for the Bharal population, with no indications of recent expansions or bottlenecks.

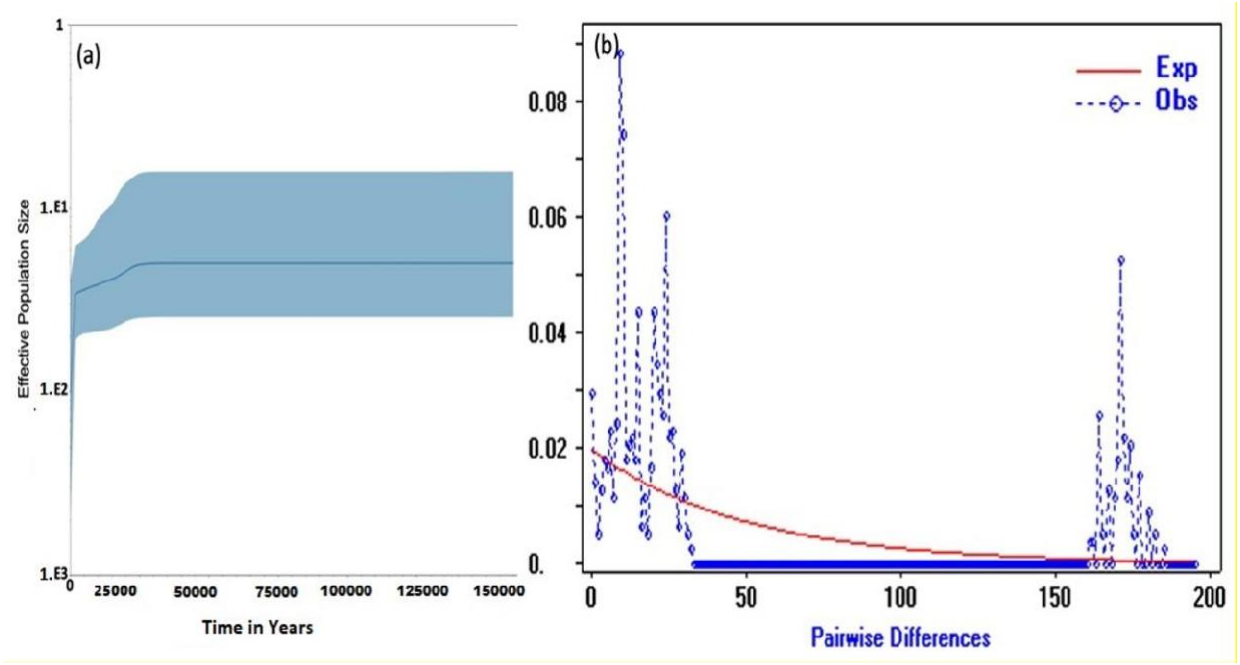

Fig. 4.2 Bayesian Skyline Plot

- **Amova Analysis**

The Analysis of Molecular Variance (AMOVA) shows that individuals (66%) had the largest proportion of genetic variation in *Pseudois nayaur* populations, therefore suggesting considerable heterozygosity at the individual level (table 4.4 & fig 4.3). Variation among populations suggests modest genetic difference between groups as 22% of the total genetic variance can be explained by it. In addition, 12% of the variance among Bharal living in populations reflects some level of genetic diversity inside every community. These findings underline the need of preserving genetic variety at both the population and individual levels as most genetic variation persists inside Bharal even if population structure exists.

Table 4.4: Genetic Variation Partitioned Among Populations, Individuals, and Within Individuals

Source	df	SS	MS	Est. Var.	%
Among Pops	1	35.615	35.615	0.770	22%
Among Indiv	45	140.853	3.130	0.411	12%
Within Indiv	47	108.500	2.309	2.309	66%
Total	93	284.968	41.054	3.489	100%

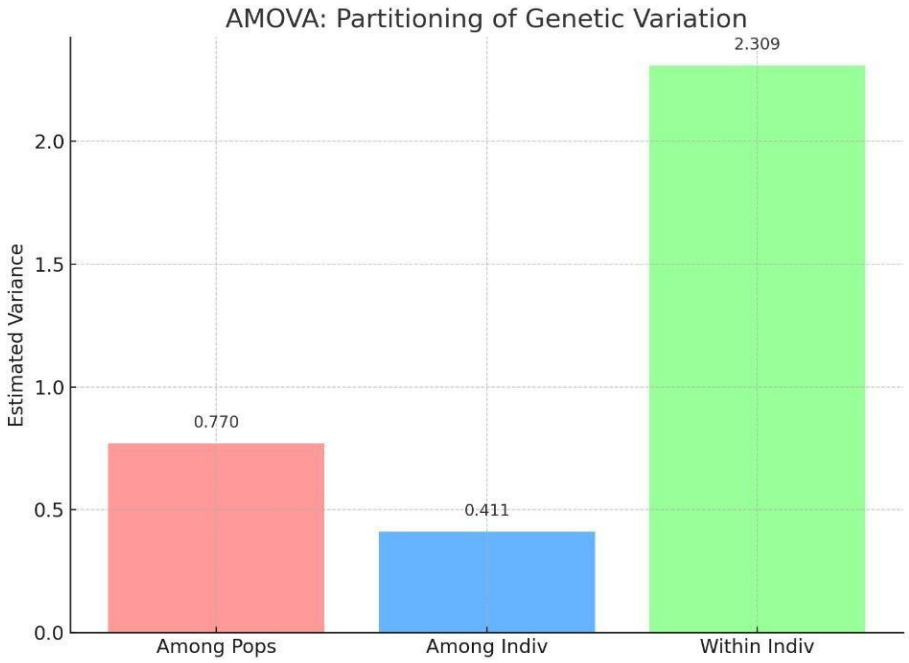

Fig. 4.3 Genetic Variation Partitioned Among Populations, Individuals, and Within Individuals

- **Population genetic structure and genetic differentiation**

Figure 4.4 depicts the correlation between the Bayesian Information Criterion (BIC) and the quantity of clusters in a clustering study, wherein a diminished BIC signifies a more ideal model that balances fit and complexity. A notable fall in BIC is noted when the number of clusters rises from 1 to 2, then followed by a progressive reduction till five clusters. The ideal BIC value occurs at four clusters, showing that this arrangement achieves the best balance between fit and simplicity. Beyond four clusters, BIC values show slight variations without a consistent decrease, suggesting that further clusters either could cause overfitting or not significantly improve model quality. Four is the optimal number of clusters for the dataset as it offers the best balance between explanatory' efficacy and complexity.

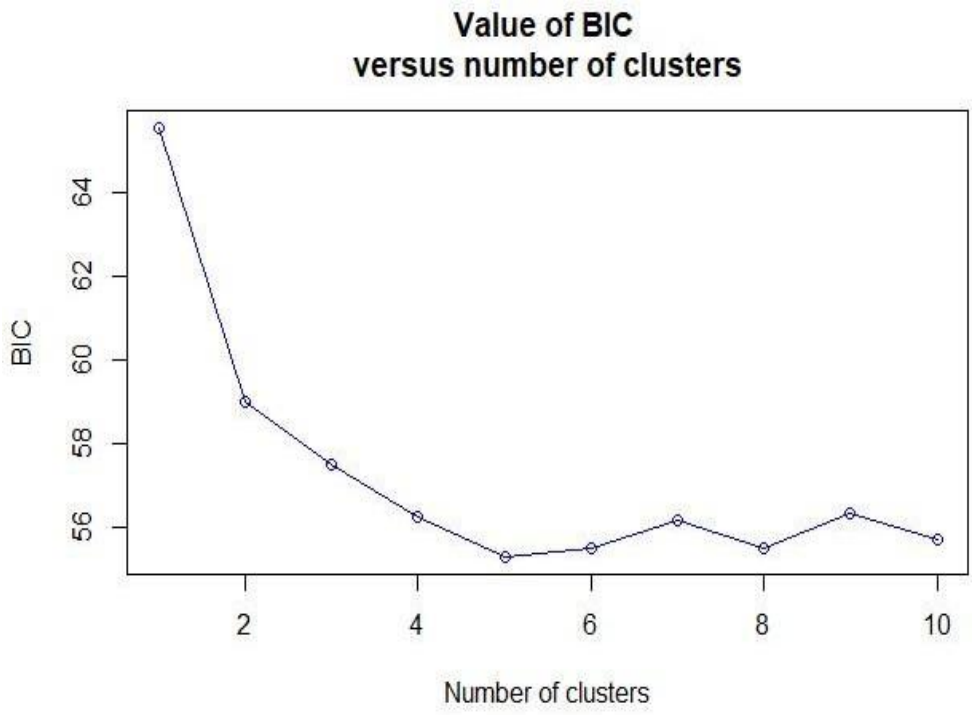

Fig. 4.4 The relationship between the number of clusters and the Bayesian Information Criteria (BIC)

Figure 4.5 illustrates a non-bayesian clustering Discriminant Analysis of Principal Components (DAPC) plot, a multivariate technique frequently utilized in population genetics to depict genetic diversity within established groups by integrating Principal Component Analysis (PCA) and Discriminant Analysis (DA). This scatterplot displays individual points, with ellipses indicating the dispersion and central tendency of the four genetic groups, designated 1 to 4, inside the reduced multivariate space. The figure demonstrates distinct genetic architecture among the groupings showing significant genetic difference, whereas Cluster 2 (brown) represent the eastern Himalaya population and also exhibits pronounced separation, implying historical isolation or restricted gene flow. Conversely, Clusters 1 (blue), 3 (orange), and Clusture 4 (brown) represent the western Himalaya population exhibit partial overlap, indicating a level of genetic closeness or recent gene flow between them. The DA eigenvalues barplot (top left) demonstrates the variance percentage elucidated by the discriminant functions, while the PCA eigenvalues plot (top right) indicates the contribution of the principal components included in the study. The pronounced geographic segregation among the clusters indicates a substantial genetic

structure among the tested populations, providing insights into their evolutionary history, migratory patterns, and possible impediments to gene flow.

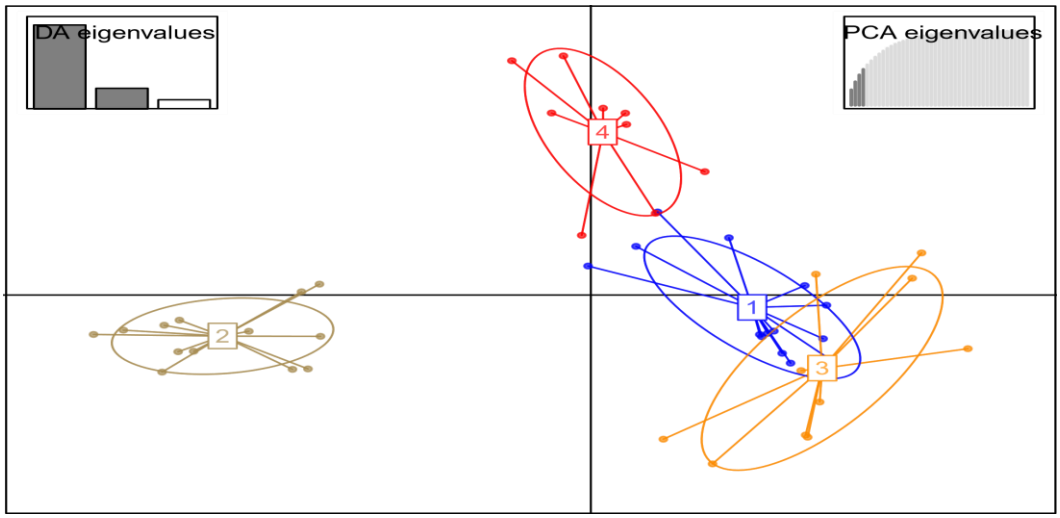

Fig. 4.5 Non-Bayesian pattern of Bharal with DAPC

Figure 4.6 Bayesian clustering depicts the results of a population structure analysis for two groups, WH and EH, using varying numbers of genetic clusters (K = 2 to K = 6). A significant genetic difference is shown at K=2; WH is mostly connected to the red cluster and EH to the green cluster, therefore indicating an obvious division between the two groups. Further substructure within WH becomes obvious when K increases to 3 and 4, with the appearance of additional colour components (blue and yellow), therefore highlighting genetic structure in this group. With K=5, the structure gets more complex, as several individuals have mixed ancestry, indicating admixture events or more nuanced genetic difference. At K=6, the complexity of the population structure escalates, especially within WH, with the formation of additional clusters (e.g., pink and cyan), but EH stays predominantly homogenous, consistently characterized by a singular cluster (green or cyan). These data indicate that the EH group retains genetic homogeneity, but the WH group displays significant genetic variation, potentially reflecting historical mixing or more extensive genetic origins. The ideal value of K is generally ascertained by model selection criteria, such as BIC or ΔK, ʿto reconcile the model's complexity with its explanatory efficacy.

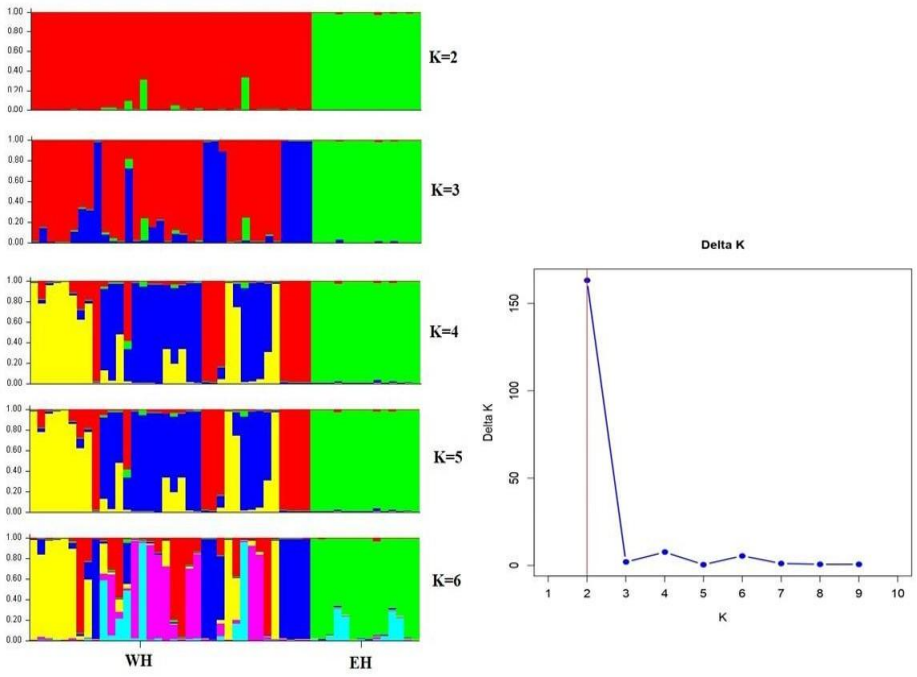

Fig. 4.6. Bayesian clustering patterns of Bharal population at K2,K3,K4,K5,K6; Assignment at population level (cluster 1: Western Himalaya, cluster 2: Easter Himalaya); A (i): Mean L (K), A (ii) The ad hoc quantity (delta K) over 20 runs for each K value. The structure analysis was performed using STRU CTU RE v 2.3.4.

○ Analysis of Response Curves for Environmental Variables

The response curves for critical parameters affecting the distribution of Bharal were delineated individually for the WH and Sikkim state in the EH region (Fig. 4.7). The minimum temperature of the coldest month (40.4%), elevation (19.9%), and maximum temperature of the hottest month (16.5%) substantially influenced the habitat selection of *P. nayaur* in WH. This species is restricted to exclusive, permanent, and secure habitats at elevated altitudes in mountains with little vegetation. Conversely, both factors, namely the minimum temperature of the coldest month (35.2%) and the maximum temperature of the warmest month (25.5%), significantly influenced distribution in the EH region, along with precipitation seasonality (13.6%), and played a crucial role in habitat selection of *P. nayaur* in Sikkim.

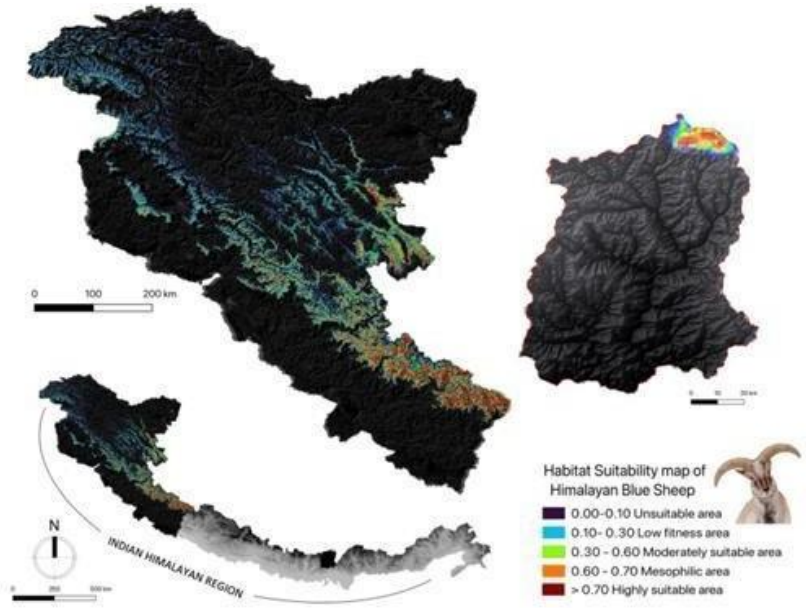

Fig. 4.7 Map of the suitable distribution area of Bharal in Western Himalayas and Sikkim (Eastern Himalayas) (MaxEnt v3.4.4)

The figure 4.8 presents a heatmap that delineates the correlation between genetic distance and geographic distance (quantified in kilometers), frequently employed to investigate isolation by distance across populations. Each point is a pairwise comparison of individuals or groups, with the color gradient (from blue to red) denoting the density of observations— red suggesting greater densities and blue indicating lower densities. The heatmap indicates two separate high-density areas: one at short geographic distances (0–200 km) and another at greater distances (~1000–1200 km), both demonstrating increased genetic distances (about 5.5 to 6.5). These trends indicate that groups separated by both short and vast geographic distances may exhibit significant genetic diversity. The significant genetic distances seen across short geographic separations may suggest pronounced genetic structuring at confined geographical scales, either resulting from historical impediments to gene flow or local adaption. In contrast, the center portion of the figure (400–800 km) has diminished densities and decreased genetic distances, potentially indicating fewer pairwise comparisons or more uniform genetic links within this range. This discontinuity may signify a genetic interruption or a sampling deficiency. The findings indicate a clear isolation-by-distance trend, A genetic distance test (IBD) result indicated weak connectivity between WH and EH populations, signifying substantial genetic divergence (Fig 4.7). This divergence is likely due to variations

in bioclimatic conditions and topography, which have shaped distinct ecological niches for these populations (Ranjitsinh 2017; Joshi et al. 2022).

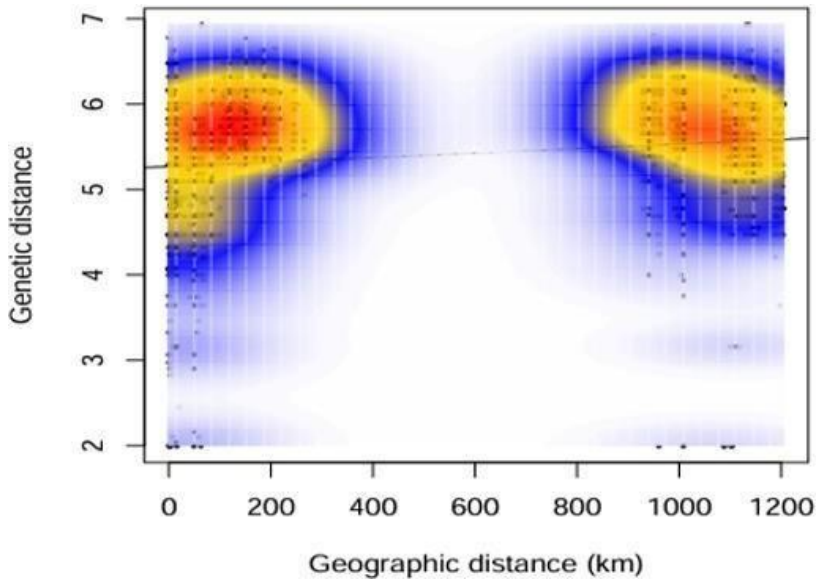

Figure 4.8 Scatterplot showing the result of Mantel test for presence of IBD (Isolation by distance) between significance of geographical distance on the genetic distance (r = 0.381) in the Bharal population. Colour represent the relative density of point higher densities are represented by warmer colour while show the correlation between the distance matrices. IBD was performed in R.4.0 software with Adegenet version 1.3.4 package R.

4.4 Discussion

This study examined the genetic structure, demographic history, and environmental preferences of *Pseudois nayaur* (Bharal) populations across the western Himalayas (WH) and eastern Himalayas (EH) using complete mitochondrial genome sequences and microsatellite markers. The findings reveal pronounced population structuring, varying levels of genetic differentiation, and ecological divergence, offering important insights into the species' evolutionary history and conservation needs.

Pairwise genetic differentiation based on mitochondrial sequences indicated moderate to high divergence among sampled individuals. The greatest genetic distance (4.1%) occurred between Mitochondrial_genome_India_1 and JX101653.1_*P. nayaur*, suggesting substantial divergence, whereas the lowest differentiation (1.9%) was observed among *P. n. szechuanensis* sequences and

NC_020632.1_*P. nayaur*, indicating close genetic affinity. These results support the presence of two distinct lineages: the WH Himalayan Bharal (*P. n. nayaur*) and the EH–Tibetan Chinese Bharal (*P. n. szechuanensis*), consistent with earlier studies (Tan et al., 2012; Gao et al., 2020). The relatively low divergence within the *P. n. szechuanensis* clade, coupled with their tight phylogenetic clustering, suggests either recent shared ancestry or ongoing gene flow among EH populations.

Analysis of molecular variance showed that 66% of genetic variation occurred within individuals, 22% among populations, and 12% within populations, highlighting considerable within-individual genetic diversity. This pattern is comparable to that reported for other Himalayan Caprinae (Shrestha & Wegge, 2008; Aryal et al., 2015) and suggests moderate gene flow with localized differentiation, likely influenced by habitat fragmentation and rugged topography(Fig. 4.3).

STRUCTURE and DAPC analyses revealed clear separation between WH and EH populations, most apparent at K = 2 and K = 5. EH individuals consistently formed a single genetic cluster, indicating homogeneity, whereas WH populations exhibited substantial admixture and higher genetic diversity—patterns consistent with broader historical gene flow, past population expansion, or secondary contact zones. The haplotype network further reflected this structure, with WH populations showing more interconnected haplotypes, potentially representing ancestral lineages.

Ecological niche modelling and response curve analysis indicated distinct environmental preferences between the regions. In WH, the minimum temperature of the coldest month (40.4%) and elevation (19.9%) were the strongest predictors of habitat suitability, pointing to adaptations to cold, high-altitude conditions with minimal human disturbance. In contrast, EH (Sikkim) populations were influenced by both temperature and precipitation seasonality (13.6%), suggesting greater ecological plasticity but also sensitivity to seasonal climatic variation (Thapa et al., 2011)(Fig. 4.7). These differences indicate ecological divergence driven by regional bioclimatic conditions. Projected climate change—particularly rising temperatures and altered precipitation regimes—may shift species distributions and population dynamics, underscoring the need for region-specific conservation planning.

Neutrality tests using Tajima's D and Fu's Fs provided no strong evidence for recent demographic expansion in any population. *P. n. nayaur* showed slightly positive

Tajima's D values and significantly negative Fu's Fs, consistent with historical stability rather than recent growth. *P. n. szechuanensis* populations yielded positive but non-significant values, also suggesting demographic stability. These results align with earlier findings, supporting long-term equilibrium in Himalayan Bharal populations.

The Mantel test revealed a moderate correlation (r = 0.381) between genetic and geographic distances, indicating isolation by distance (IBD) across the species' range. Combined with STRUCTURE and DAPC patterns, this supports the conclusion of limited gene flow and substantial geographic structuring, shaped by topographic complexity and historical climatic fluctuations (Ranjitsinh, 2017)(Fig 4.6).

In summary, this study presents strong evidence of significant genetic divergence, population structure, and ecological differentiation in *Pseudois nayaur*. The findings support the recognition of two Evolutionarily Significant Units, *P. n. nayaur* and *P. n. szechuanensis* and reinforce the importance of tailored, region-specific conservation strategies to maintain genetic diversity and adaptive potential. Future work employing genome-wide SNP datasets, ancient DNA, and landscape genetic modelling will be critical for refining conservation priorities and assessing adaptive capacity under accelerating environmental change in the Himalayas.

Chapter-5

Summary of Results

The genus *Pseudois* serves as an important model for understanding the evolutionary history and phylogeographic structuring of high-altitude ungulates in the Himalayan region. *Pseudois nayaur* (Bharal) is currently recognized as a single, widely distributed species inhabiting parts of China, the Indian Himalayas, and the Tibetan Plateau. However, previous research has shown that under the combined influence of complex topography, ecological heterogeneity, and historical climatic oscillations, *P. nayaur* exhibits pronounced genetic variation and potentially cryptic lineages across its range.

The present study aimed to: (i) investigate the phylogenetic relationships of *P. nayaur* with other bovids; (ii) assess genetic diversity within Indian populations using mtDNA and microsatellite markers; and (iii) evaluate population genetic structure, gene flow, and demographic history across the Indian Himalayan Region (IHR). Chapter 1 introduced the ecological significance, distribution, and unresolved taxonomic issues surrounding *P. nayaur*. Chapter 2 explored phylogenetic relationships and phylogeographic structuring, revealing two major clades corresponding to the western Himalayas (WH) and eastern Himalayas (EH), with molecular clock analyses indicating divergence during the mid-Pleistocene. Chapter 3 examined genetic diversity patterns, identifying considerable within-population variability and region-specific trends. Chapter 4 addressed demographic history, gene flow, and population genetic structure, revealing evidence of sub structuring, restricted recent expansion, and ecological niche variation linked to elevation and temperature.

Overall, the findings provide strong evidence for the existence of evolutionarily significant units (ESUs) within *P. nayaur*, underscoring the need for region-specific conservation measures. These results contribute valuable genetic and ecological insights that may inform future taxonomic reassessments and conservation strategies for high-altitude species threatened by climate change and habitat fragmentation.

Objective 1 - Phylogenetic relationships

As presented in Chapter 2, this objective examined the phylogenetic relationships and phylogeographic structure of *P. nayaur* across the Himalayas and adjacent regions using whole mitochondrial genome sequences and population-level sequencing. The mitogenome of *P. nayaur* consists of 13 protein-coding genes, 22 tRNAs, 2 rRNAs, and a control region, consistent with standard vertebrate mitochondrial architecture. Base composition analysis revealed AT bias and strand-specific nucleotide skews, patterns similar to other Caprinae species and reflective of mutational and replication pressures.

Phylogenetic reconstruction based on the control region and cytochrome *b* genes resolved two well-supported clades—WH and EH (Sikkim)—showing marked genetic divergence. The WH clade displayed the greatest separation from EH populations, likely due to restricted historical gene flow imposed by physical and climatic barriers. Molecular clock estimates date the divergence between *P. n. nayaur* and *P. n. szechuanensis* in the IHR to ~1.38 million years ago, during the mid-Pleistocene—a period of pronounced environmental shifts and geographic isolation in the Himalayas. Bayesian species delimitation consistently supported the existence of at least two genetically distinct lineages, indicating cryptic diversity and the possible need for taxonomic revision, including recognition of regionally restricted forms such as *P. n. szechuanensis* and *P. n. nayaur*. In light of increasing anthropogenic pressures, habitat fragmentation, and climate change, these genetic units highlight the importance of lineage-specific conservation approaches.

The results from this objective are critical for understanding the evolutionary background and genetic structure of *P. nayaur* and provide a reference for future taxonomic, ecological, and conservation-focused research on Himalayan Mountain ungulates.

Objective 2 - Genetic diversity

As outlined in Chapter 3, this objective assessed the genetic structure and diversity of *P. nayaur* populations in the Indian Himalayas, revealing substantial regional heterogeneity shaped by evolutionary, ecological, and anthropogenic factors. While

earlier studies relied on a limited number of molecular markers, this work integrated nuclear (microsatellite) and mitochondrial (cytochrome *b* and D-loop) markers, enabling the reconstruction of complex population histories. WH populations, particularly in Ladakh and Himachal Pradesh, exhibited higher mitochondrial diversity, suggestive of long-term stability, while certain groups showed reduced diversity, likely due to recent demographic events or selective pressures. In contrast, EH populations, especially in Sikkim, displayed low genetic diversity and signs of demographic contraction. Physical barriers and habitat fragmentation were found to impede gene flow.

Microsatellite analysis confirmed and refined mitochondrial results, detecting significant allelic richness and heterozygosity in EH populations. The combined findings highlight the existence of possible ESUs in *P. nayaur* with each facing threats from habitat encroachment, climate change, and livestock competition. Incorporating genetic data into conservation planning is essential to safeguard the species' adaptive potential and long-term viability across the Himalayan landscape.

Objective 3 - Population genetic structure, gene flow, and demographic history

Chapter 4 provides a comprehensive evaluation of *P. nayaur*'s population genetic structure, gene flow, and demographic history in the IHR. Significant genetic variation was observed both within and between populations, with pronounced differences between WH and EH. WH populations exhibited clear sub structuring, as shown by multivariate analyses and Bayesian clustering, along with high diversity and evidence of admixture. Conversely, EH populations reflected long-term isolation, low diversity, and limited gene flow.

Demographic analyses indicated stability in WH populations, with no evidence of recent expansion, while an isolation-by-distance pattern suggested that topographic complexity and environmental heterogeneity are key drivers of genetic structuring. Ecological niche modelling further revealed regional differences in habitat suitability, strongly influenced by elevation, temperature extremes, and precipitation seasonality factors critical to *P. nayaur*'s distribution and habitat preferences.

These results have direct conservation implications, providing strong evidence for ESUs within *P. nayaur*. Recognizing region-specific genetic lineages is essential for

developing locally tailored management strategies that account for ecological context and evolutionary distinctiveness. Conserving genetic diversity and adaptive capacity will be vital for ensuring the species' persistence under growing human pressures and climate change in high-altitude ecosystems.

Bibliography

Aryal, A., Brunton, D. and Raubenheimer, D., 2013. Habitat assessment for the translocation of Bharal to maintain a viable snow leopard population in the Mt Everest Region, Nepal. Zoology and Ecology, 23(1), pp.66-82.

Avise, J.C., 2000. Phylogeography: the history and formation of species. Harvard university press.

Avise, J.C., 2012. Molecular markers, natural history and evolution. Springer Science & Business Media.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. and Saunders, N.C., 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual review of ecology and systematics, pp.489-522.

Awasthi, A., Uniyal, S. K., Rawat, G. S., & Sathyakumar, S. (2003). Food plants and feeding habits of Himalayan ungulates. Current Science, 719-723.

Bagchi, S., 2009. Ecology of the Trans-Himalayan grazing ecosystem. Syracuse University.

Baillie, I. C., & Norbu, C. (2004). Climate and other factors in the development of river and interfluve profiles in Bhutan, Eastern Himalayas. Journal of Asian Earth Sciences, 22(5), 539-553.

Ballard, J.W.O. and Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Molecular ecology, 13(4), pp.729-744.

Beall, C.M. and Goldstein, M.C., 1992. High prevalence of excess fat and central fat patterning among Mongolian pastoral nomads. American Journal of Human Biology, 4(6), pp.747-756.

Belkhir, K., 2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. http://www. genetix. univ-montp2. fr/genetix/genetix. htm.

Bernt, M., Donath, A., Jühling, F., Externbrink, F., Florentz, C., Fritzsch, G., ... & Stadler, P. F. (2013). MITOS: improved de novo metazoan mitochondrial genome annotation. Molecular phylogenetics and evolution, 69(2), 313-319.

Bhatnagar, Y.V., Wangchuk, R. and Mishra, C., 2006. Decline of the Tibetan gazelle Procapra picticaudata in Ladakh, India. Oryx, 40(2), pp.229-232.

Bhatt, S., Biswas, S., Karanth, K., Pandav, B. and Mondol, S., 2020. Genetic analyses reveal population structure and recent decline in leopards (Panthera pardus fusca) across the Indian subcontinent. PeerJ, 8, p.e8482.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. and Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends in ecology & evolution, 22(3), pp.148-155.

Biswas, T., Subramanian, S., Niyogi, R., Mylliemngap, W., Mitra, S., Das, D.K. and Sarkar, M.S., 2025. Conservation Challenges and Adaptation Strategies for Indian Himalayan Biodiversity in a Changing Climate. In Climate Change Impact on Himalayan Biodiversity (pp. 179-224). Cham: Springer Nature Switzerland.

Brown, G. G., Gadaleta, G., Pepe, G., Saccone, C., & Sbisà, E. (1986). Structural conservation and variation in the D-loop-containing region of vertebrate mitochondrial DNA. Journal of molecular biology, 192(3), 503-511.

Buzzard, P. J., Li, X., & Bleisch, W. V. (2018). High altitude ungulate communities in southwest China. Mammalia, 82(5), 415-422.

Cancellare, I.A., 2023. Phylogeography, population structure, and landscape genetics of snow leopards across High Asia. University of Delaware.

Chang, E., Shi, L., Zhou, R., Zhang, J., Ma, H., & Wan, Q. (2012). Species and gender identification of sympatric deer species in China using a sex- and species-specific multiplex

Chen, A.J., Suchard, M.A., Xie, D. and Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Molecular biology and evolution, 29(8), pp.1969-1973.

Choudhury, A., Aron, S., Sengupta, D., Hazelhurst, S. and Ramsay, M., 2018. African genetic diversity provides novel insights into evolutionary history and local adaptations. Human molecular genetics, 27(R2), pp.R209-R218.

Conservation biology, 10(6), pp.1500-1508.

Coogan, S. C., Raubenheimer, D., Stenhouse, G. B., & Nielsen, S. E. (2014). Macronutrient optimization and seasonal diet mixing in a large omnivore, the grizzly bear: a geometric analysis. PloS one, 9(5), e97968.

Cun, Y.Z. and Wang, X.Q., 2010. Plant recolonization in the Himalaya from the southeastern Qinghai-Tibetan Plateau: Geographical isolation contributed to high population differentiation. Molecular Phylogenetics and Evolution, 56(3), pp.972-982.

Dolker, S., Jabin, G., Singh, S.K., Joshi, B.D., Singh, V.K., Dalui, S., Chandra, K., Sharma, L.K. and Thakur, M., 2023. Landscape genetics identified conservation priority areas for Bharal (Pseudois nayaur) in the Indian Trans-Himalayan Region. Scientific Reports, 13(1), p.18152.

Doolittle, W.F., 1999. Phylogenetic classification and the universal tree. Science, 284(5423), pp.2124-2128.

Dou, H., Zhang, Y. and Feng, L., 2016. Complete mitochondrial genome of the Himalayan serow (Capricornis thar) and its phylogenetic status within the genus Capricornis. Biochemical Systematics and Ecology, 65, pp.115-123.

Drummond, A.J., Rambaut, A., Shapiro, B.E.T.H. and Pybus, O.G., 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Molecular biology and evolution, 22(5), pp.1185-1192.

Dutta, R., Kumar, V., Sharief, A., Singh, H., Joshi, B.D., Thakur, M., Sharma, L.K. and Babu,

E.C., 2004. Unifying the epidemiological and evolutionary dynamics of pathogens. science, 303(5656), pp.327-332.

Earl, D.A. and VonHoldt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation genetics resources, 4, pp.359-361.

Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular ecology resources, 10(3), 564-567.

Fahrig, L., 2003. Effects of habitat fragmentation on biodiversity. Annual review of ecology, evolution, and systematics, 34(1), pp.487-515.

Faith, D.P., 1992. Conservation evaluation and phylogenetic diversity. Biological conservation, 61(1), pp.1-10.

Felsenstein, J., 2004. Inferring phylogenies Sinauer Associates Inc. Sunderland, MA. xx+. pp.

Feng, B., Xiao, Y., Hu, L., Yang, X., Dong, X., Zhang, J., ... & Bai, W. (2024). Predicted Climate Change Impacts on Distribution and Habitat Structure of Forest Ungulates in Southwest China. Ecosystem Health and Sustainability, 10, 0173.

Fletcher, N.K., 2020. Patterns of Genomic Diversity within and between Cryptic Species of Field Voles and Surfclams (Doctoral dissertation, Cornell University).

Fox, J.L., Sinha, S.P., Chundawat, R.S. and Das, P.K., 1991. Status of the snow leopard Panthera uncia in northwest India. Biological conservation, 55(3), pp.283-298.

Frankham, R., 1996. Relationship of genetic variation to population size in wildlife.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics, 147(2), pp.915-925.

Gao, H. et al. Taxonomic status of Chinese Bharal (Pseudois nayaur): New evidence of a distinct subspecies. Integr. Zool. 15, 202–212 (2020).

Garant, D., 2007. Hartl, DL & AG Clark, 2007. Principles of Population Genetics.

Gaur, A., Singh, L., & Kumar, S. (2003). Polymorphic microsatellites in the Indian bison (Bos gaurus). Molecular Ecology Notes, 3(1), 83–84. https://doi.org/10.1046/j.1471-

Ge, R. L., Cai, Q., Shen, Y. Y., San, A., Ma, L., Zhang, Y., ... & Wang, J. (2013). Draft genome sequence of the Tibetan antelope. Nature communications, 4(1), 1858.

Ghalib, S.A., Khan, M.Z., Kanwal, R., Zehra, A., Siddiqui, S., Abbas, D., Yasmeen, G., Hussain, B. and Khan, A.R., 2019. Recent observations on the distribution and status of wildlife of Baluchistan. Canadian Journal of Pure and Applied Sciences, 13(2), pp.48134846.

Glaubitz, J.C., 2004. Convert: a user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes, 4(2), pp.309-310.

Goudet, J. (2002). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9. 3.2). http://www. unil. ch/izea/softwares/fstat. html.

Grenfell, B.T., Pybus, O.G., Gog, J.R., Wood, J.L., Daly, J.M., Mumford, J.A. and Holmes,

Groves, C. and Grubb, P., 2011. Ungulate taxonomy John Hopkins University Press. Baltimore, USA.

Groves, P. and Shields, G.F., 1996. Phylogenetics of the Caprinae based on cytochrome b sequence. Molecular Phylogenetics and Evolution, 5(3), pp.467-476.

Gupta, S. K., Kumar, A., & Hussain, S. A. (2013). Extraction of PCR-amplifiable DNA from a variety of biological samples with uniform success rate. Conservation genetics resources, 5(1), 215-217.

Hall, T.A., 1999, January. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series (Vol. 41, No.

Harris, R.B., 2014. Pseudois nayaur. The IUCN Red List of Threatened Species.

Hassanin, A. and Douzery, E.J., 2003. Molecular and morphological phylogenies of Ruminantia and the alternative position of the Moschidae. Systematic biology, 52(2), pp.206-228.

Hassanin, A., Delsuc, F., Ropiquet, A., Hammer, C., Van Vuuren, B.J., Matthee, C., RuizGarcia, M., Catzeflis, F., Areskoug, V., Nguyen, T.T. and Couloux, A., 2012. Pattern and timing of diversification of Cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes. Comptes rendus biologies, 335(1), pp.3250.

Hebert, P. D., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512), 313-321.

Heinen, J.T. and Kattel, B., 1992. Parks, people, and conservation: a review of management issues in Nepal's protected areas. Population and Environment, 14, pp.49-84.

Hewitt, G., 2000. The genetic legacy of the Quaternary ice ages. Nature, 405(6789), pp.907913.

Hibbett, D.S., Binder, M., Bischoff, J.F., Blackwell, M., Cannon, P.F., Eriksson, O.E., Huhndorf, S., James, T., Kirk, P.M., Lücking, R. and Lumbsch, H.T., 2007. A higher-level phylogenetic classification of the Fungi. Mycological research, 111(5), pp.509-547.

Hillis, D.M., 1987. Molecular versus morphological approaches to systematics. Annual review of Ecology and Systematics, pp.23-42.

Himalayan ibex to evolve independently after split into an allopatric refugium. Biology, 12(8), p.1097.

Ho, S.Y. and Larson, G., 2006. Molecular clocks: when timesare a-changin'. TRENDS in Genetics, 22(2), pp.79-83.

Hulce, D., Li, X., Snyder-Leiby, T. and Liu, C.J., 2011. GeneMarker® genotyping software: Tools to increase the statistical power of DNA fragment analysis. Journal of biomolecular techniques: JBT, 22(Suppl), p.S35.

Irwin, D.M., Kocher, T.D. and Wilson, A.C., 1991. Evolution of the cytochrome b gene of mammals. Journal of molecular evolution, 32, pp.128-144.

Jabin, G., Joshi, B.D., Wang, M.S., Mukherjee, T., Dolker, S., Wang, S., Chandra, K., Chinnadurai, V., Sharma, L.K. and Thakur, M., 2023. Mid-Pleistocene transitions forced

Jarne, P. and Lagoda, P.J., 1996. Microsatellites, from molecules to populations and back.

Jombart, T., Devillard, S. and Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC genetics, 11, pp.1-

Jones, K.C., Levine, K.F. & Banks, J.D. (2000). Characterization of 10 polymorphic microsatellite loci in the mule deer (Odocoileus hemionus). Molecular Ecology, 9(2), 283–

Joshi, B. D., Kumar, V., De, R., Sharma, R., Bhattacharya, A., Dolker, S., ... & Goyal, S. P. (2022). Mitochondrial cytochrome b indicates the presence of two paraphyletic diverged lineages of the blue sheep Pseudois nayaur across the Indian Himalaya: conservation implications. Molecular Biology Reports, 49(11), 11177-11186.

Kalinowski, S. T., Taper, M. L. & Marshall, T. C. Revising how the computer program cervus accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16, 1099–1106 (2007).

Karmacharya, A., 2022. Molecular Mapping of QTL for Genetic Transformation-Related Trait and CRISPR/Cas9-Mediated Gene Editing in Wheat (Doctoral dissertation, North Dakota State University).

Kim, C., Deng, T., Wen, J., Nie, Z.L. and Sun, H., 2015. Systematics, biogeography, and character evolution of Deutzia (Hydrangeaceae) inferred from nuclear and chloroplast DNA sequences. Molecular Phylogenetics and Evolution, 87, pp.91-104.

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.

Kundu, S., Chandra, K., Tyagi, K., Pakrashi, A. and Kumar, V., 2019. DNA barcoding of freshwater fishes from Brahmaputra River in Eastern Himalaya biodiversity hotspot.

Lemey, P., Salemi, M. and Vandamme, A.M. eds., 2009. The phylogenetic handbook: a practical approach to phylogenetic analysis and hypothesis testing. Cambridge University Press.

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

Lohse, M., Drechsel, O., Kahlau, S., & Bock, R. (2013). OrganellarGenomeDRAW—a suite of tools for generating physical maps of plastid and mitochondrial genomes and visualizing expression data sets. Nucleic acids research, 41(W1), W575-W581.

Lü, X., Hou, X., & Han, X. (2000). Grazing impact on plant diversity in alpine meadows. Acta Phytoecologica Sinica, 24(1), 123–130.

Maddison, D.R. ed., 2001. The tree of life web project.

Mallet, J., 2005. Hybridization as an invasion of the genome. Trends in ecology & evolution, 20(5), pp.229-237.

Mallon, D. P. (1991). Status and conservation of large mammals in Ladakh. Biological Conservation, 56(1), 101-119.

Manceau, V., Després, L., Bouvet, J. and Taberlet, P., 1999. Systematics of the genus Capra inferred from mitochondrial DNA sequence data. Molecular Phylogenetics and Evolution, 13(3), pp.504-510.

Mani, M.S., 1974. In MS Mani. Ecology and biogeography in India, 23, pp.1-808.

Martens, J., Tietze, D.T. and Päckert, M., 2011. Phylogeny, biodiversity, and species limits of passerine birds in the Sino-Himalayan region—a critical review. Ornithological Monographs, 70(1), pp.64-94.

Meredith, R.W., Janečka, J.E., Gatesy, J., Ryder, O.A., Fisher, C.A., Teeling, E.C., Goodbla, A., Eizirik, E., Simão, T.L., Stadler, T. and Rabosky, D.L., 2011. Impacts of the Cretaceous Terrestrial Revolution and KPg extinction on mammal diversification. science, 334(6055), pp.521-524.

Miller, D. J. (1987). Yaks and grasses: pastoralism in the Himalayan countries of Nepal and Bhutan and Strategies for sustained development.

Miller, M.P., 2005. Alleles In Space (AIS): computer software for the joint analysis of interindividual spatial and genetic information. Journal of Heredity, 96(6), pp.722-724.

Mishra, C., Van Wieren, S. E., Ketner, P., Heitkönig, I. M., & Prins, H. H. (2004). Competition between domestic livestock and wild bharal Pseudois nayaur in the Indian Trans-Himalaya. Journal of Applied Ecology, 41(2), 344-354.

Mitochondrial DNA Part B, 4(2), pp.2411-2419.

Mittermeier, R.A. and Wilson, D.E., 2011. Handbook of the mammals of the world: Vol. 2: hoofed mammals.

Molnar, P., England, P. and Martinod, J., 1993. Mantle dynamics, uplift of the Tibetan Plateau, and the Indian monsoon. Reviews of Geophysics, 31(4), pp.357-396.

Mukai, T., Vargas-Rodriguez, O., Englert, M., Tripp, H. J., Ivanova, N. N., Rubin, E. M., ... & Söll, D. (2017). Transfer RNAs with novel cloverleaf structures. Nucleic Acids Research, 45(5), 2776-2785.

Namgail, T., Fox, J.L. and Bhatnagar, Y.V., 2004. Habitat segregation between sympatric Tibetan argali Ovis ammon hodgsoni and Bharal Pseudois nayaur in the Indian Trans-Himalaya. Journal of Zoology, 262(1), pp.57-63.

Nei, M. and Kumar, S., 2000. Molecular evolution and phylogenetics. Oxford university press.

Nowak, R.M., 1999. Walker's Mammals of the World (Vol. 1). JHU press.

Nybom, H., Weising, K., & Rotter, B. (2014). DNA fingerprinting in botany: past, present, future. Investigative genetics, 5(1), 1.

Page, R.D. and Holmes, E.C., 2009. Molecular evolution: a phylogenetic approach. John Wiley & Sons.

PCR. Molecular Ecology Resources, 12(5), 822–825. https://doi.org/10.1111/j.17550998.2012.03155.x

Peakall, R. & Smouse, P. E. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research: An update. Bioinformatics 28, 2537–2539 (2012).

Petit, R.J., Duminil, J., Fineschi, S., Hampe, A., Salvini, D. and Vendramin, G.G., 2005. Invited review: comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. Molecular ecology, 14(3), pp.689-701.

Phillips, S. J., Dudík, M., & Schapire, R. E. (2010). Maxent software for species habitat modeling, version 3.3. 3.

Poetsch, M., Meyer, H. J., & Bajanowski, T. (2001). Application of DNA fingerprinting in forensic medicine. International Journal of Legal Medicine, 114(5), 202–205. https://doi.org/10.1007/s004140000187

Pritchard, J.K., Stephens, M. and Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics, 155(2), pp.945-959.

Pybus, O.G. and Rambaut, A., 2009. Evolutionary analysis of the dynamics of viral infectious disease. Nature Reviews Genetics, 10(8), pp.540-550.

Qiu, Q., Wang, L., Wang, K., Yang, Y., Ma, T., Wang, Z., Zhang, X., Ni, Z., Hou, F., Long, R. and Abbott, R., 2015. Yak whole-genome resequencing reveals domestication signatures and prehistoric population expansions. Nature communications, 6(1), p.10283.

R., 2024. Diel Activity Patterns of Himalayan Ibex and Livestock in the Trans-Himalayan Landscape of Himachal Pradesh, India. Indian Journal of Ecology, 51(1), pp.63-68.

Rana, S.K., Rana, H.K., Landis, J.B., Kuang, T., Chen, J., Wang, H., Deng, T., Davis, C.C. and Sun, H., 2023. Pleistocene glaciation advances the younger temporal dimension of species diversification in a major biodiversity hotspot. bioRxiv, pp.2023-08.

Ranjitsinh, M. K. (2017). A life with wildlife: from princely India to the present. HarperCollins.

Rawal, R. S., Bhatt, I. D., Sekar, K. C., & Nandi, S. K. (2013). The himalayan biodiversity: richness, representativeness, uniqueness and life-support values. GB Pant Institute of Himalayan Environment and Development (GBPIHED), Kosi-Katarmal, Almora, Uttarakhand, India, 84pp.

Rawat, G.S. and Adhikari, B.S., 2015. Ecology and management of grassland habitats in India. ENVIS bulletin: wildlife & protected areas, 17.

Rawat, G.S., 2005. Alpine meadows of Uttaranchal: ecology, landuse, and status of medicinal & aromatic plants. Bishen Singh Mahendra Pal Singh.

Roberts, T.J. and Bernhard (principe d'Olanda.), 1977. The mammals of Pakistan.

Rodgers, W.A. and Panwar, H.S., 1988. Planning a wildlife protected area network in India.

Saini, D., Sonker, G., Parab, T., Kolipakam, V., Sathyakumar, S., Lyngdoh, S. and Gupta, S.K., 2025. Molecular Taxonomy Suggests Presence of Two Distinct Lineages of Bharal (Pseudois nayaur) in Indian Himalayan Region. Biochemical Genetics, pp.1-18.

Salemi, M. and Vandamme, A.M. eds., 2003. The phylogenetic handbook: a practical approach to DNA and protein phylogeny. Cambridge University Press.

Sathyakumar, S. (2001). Status and management of Asiatic black bear and Himalayan brown bear in India. Ursus, 21-29.

Schaller, G.B., 1977. Mountain monarchs. Wild sheep and goats of the Himalaya (pp. 425-pp).

Schaller, G.B., 2000. Wildlife of the Tibetan steppe. University of Chicago Press.

Selkoe, K.A. and Toonen, R.J., 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecology letters, 9(5), pp.615-629.

Shackleton, D.M., 1997. Wild sheep and goats and their relatives: status survey and conservation action plan for Caprinae.

Sharief, A., Singh, V.K., Kumar, V., Singh, H., Ghosh, A., Joshi, B.D., Ramesh, C., Thakur, M. and Sharma, L.K., 2025. Range-wide understanding of genetic diversity and population structure of endangered Kashmir musk deer in North-Western Himalaya. Molecular Biology Reports, 52(1), pp.1-9.

Shrestha, R. and Wegge, P., 2008. Wild sheep and livestock in Nepal Trans-Himalaya: coexistence or competition?. Environmental Conservation, 35(2), pp.125-136.

Singh, V.K., Joshi, B.D., Dalui, S., Ghosh, A., Jabin, G., Sharief, A., Kumar, V., Singh, H., Singh, S.K., Chandra, K. and Sharma, L.K., 2023. Genetic diversity

and population structure of Himalayan tahr (Hemitragus jemlahicus) from Western Himalaya. Mammalia, 87(3), pp.238-244.

Slatkin, M., 1987. Gene flow and the geographic structure of natural populations. Science, 236(4803), pp.787-792.

Sodhi, M., Sobti, R.C. and Mukesh, M., 2022. Mitochondrial DNA: A tool for elucidating molecular phylogenetics and population. In Advances in animal experimentation and modeling (pp. 27-38). Academic Press.

Suryawanshi, K. R., Bhatnagar, Y. V., & Mishra, C. (2010). Why should a grazer browse? Livestock impact on winter resource use by bharal Pseudois nayaur. Oecologia, 162(2), 453-462.

Swofford, D.L., 1993. PAUP, phylogenetic analysis using parsimony. version 3.1. Computer program distributed by the Illinois Natural History Survey.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics, 123(3), pp.585-595.

Tamura, K., Stecher, G. and Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Molecular biology and evolution, 38(7), pp.3022-3027.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution, 30(12), pp.2725-2729.

Tan, S., Zou, D., Tang, L., Wang, G., Peng, Q., Zeng, B., Zhang, C. and Zou, F., 2012. Molecular evidence for the subspecific differentiation of Bharal (Pseudois nayaur) and polyphyletic origin of dwarf Bharal (Pseudois schaeferi). Genetica, 140, pp.159-167.

Tautz, D. and Domazet-Lošo, T., 2011. The evolutionary origin of orphan genes. Nature Reviews Genetics, 12(10), pp.692-702.

Thapa, K., Wikramanayake, E., Jnawali, S. R., Malla, S., Acharya, R., & Lamichhane, B. R. (2011). Climate change impacts on biodiversity and ecosystem services: A case study of the alpine region of Nepal. MOE/UNDP/Nepal Climate Change Support Programme.

Trends in ecology & evolution, 11(10), pp.424-429.

Tumendemberel, O., 2020. Evolutionary history, demographics, and conservation of brown bears: Filling the knowledge gap in Central Asia (Doctoral dissertation, Dissertation, University of South-Eastern Norway, Bø i Telemark, Norway).

Vaiman, D., Mercier, D., Moazami-Goudarzi, K., Eggen, A., Ciampolini, R., Lépingle, A., Velmala, R., Kaukinen, J., Varvio, S.L., Martin, P. and Levéziel, H., 1994. A set of 99 cattle microsatellites: characterization, synteny mapping, and polymorphism. Mammalian Genome, 5, pp.288-297.

Valentová, K. A., Shrestha, B., Rokaya, M. B., & Kindlmann, P. (2022). Distribution, Threats and Conservation of Snow Leopard Throughout the World. In Snow Leopards in Nepal: Predator-Prey System on the Top of the World (pp. 1-32). Cham: Springer International Publishing.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P., & Shipley, P. (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular ecology notes, 4(3), 535-538.

Vintsek, L., Klichowska, E., Nowak, A. and Nobis, M., 2022. Genetic differentiation, demographic history and distribution models of high alpine endemic vicariants outline the response of species to predicted climate changes in a Central Asian biodiversity hotspot. Ecological Indicators, 144, p.109419.

Wang, X. M., Peng, J. T., & Zhou, H. M. (2000). Preliminary observations on the distribution and status of dwarf blue sheep Pseudois schaeferi. Oryx, 34(1), 21-26.

Wei, C., Wang, H., Liu, G., Zhao, F., Kijas, J.W., Ma, Y., Lu, J., Zhang, L.I., Cao, J., Wu, M. and Wang, G., 2016. Genome-wide analysis reveals adaptation to high altitudes in Tibetan sheep. Scientific reports, 6(1), p.26770.

White, J. C. (1909). Sikhim and Bhutan. London.

Wiens, J.J. and Penkrot, T.A., 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (Sceloporus). Systematic biology, 51(1), pp.69-

Wilberg, M. J. & Dreher, B. P. genecap: A program for analysis of multilocus genotype data for non-invasive sampling and capturerecapture population estimation. Mol. Ecol. Notes 4, 783– 785 (2004).

Wilson, D.E. and Reeder, D.M. eds., 2005. Mammal species of the world: a taxonomic and geographic reference (Vol. 1). JHU press.

Wilson, D.E., Wilson, D.E. and Mittermeier, R.A., 2011. Handbook of the mammals of the world, volume 2: hoofed mammals (pp. 886-886). Barcelona, Spain: Lynx Ediciones.

Wolf, C. and Ripple, W.J., 2016. Prey depletion as a threat to the world's large carnivores. Royal Society open science, 3(8), p.160252.

Yadav, P., Kumar, A., Hussain, S.A. and Gupta, S.K., 2020. Evaluation of the effect of longitudinal connectivity in population genetic structure of endangered golden mahseer, Tor putitora (Cyprinidae), in Himalayan rivers: Implications for its conservation. Plos one, 15(6), p.e0234377.

Yang, C., Xiang, C., Qi, W., Xia, S., Tu, F., Zhang, X., Moermond, T. and Yue, B., 2013. Phylogenetic analyses and improved resolution of the family Bovidae based on complete mitochondrial genomes. Biochemical Systematics and Ecology, 48, pp.136-143.

Yang, Z. and Rannala, B., 2012. Molecular phylogenetics: principles and practice. Nature reviews genetics, 13(5), pp.303-314.

Zeng, B., Xu, L., Yue, B., Li, Z. and Zou, F., 2008. Molecular phylogeography and genetic differentiation of Bharal Pseudois nayaur szechuanensis and Pseudois schaeferi in China. Molecular Phylogenetics and Evolution, 48(2), pp.387-395.

Zhao, X., Wang, J., Su, J., Sun, W. and Meng, H., 2021. Research on a biodiversity conservation value assessment method based on habitat suitability of species: a case study in Gansu Province, China. Sustainability, 13(6), p.3007.

Zhisheng, A., Kutzbach, J.E., Prell, W.L. and Porter, S.C., 2001. Evolution of Asian monsoons and phased uplift of the Himalaya–Tibetan plateau since Late Miocene times. nature, 411(6833), pp.62-66.

Zizka, A., Silvestro, D., Andermann, T., Azevedo, J., Duarte Ritter, C., Edler, D., ... & Antonelli, A. (2019). CoordinateCleaner: Standardized cleaning of occurrence records from biological collection databases. Methods in Ecology and Evolution, 10(5), 744-751.

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The complete mitogenome of blue sheep (*Pseudois nayaur*) from the Indian Himalayan Region and its comparative phylogenetic relationship with other related species

Deepesh Saini, Prabhaker Yadav, Vishnupriya Kolipakam, Sambandam Sathyakumar, Sandeep Kumar Gupta *

Wildlife Institute of India, Chandrabani, Dehradun 248001, Uttarakhand, India

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ABSTRACT

The '*Bharal*' or 'Himalayan Blue Sheep' (*Pseudois nayaur*) is endemic to the Himalayan and Tibetan Regions. There are gaps in the available database for the blue sheep mitogenome sequencing from the Indian region. We sequenced and characterized the whole mitogenome of one blue sheep individual using the Illumina Nova-seq 6000 platform, which was 16,718 bp in length. It included 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs), and one non-coding control region (D loop). It was compared with other complete mitochondrial DNA sequences of blue sheep from the NCBI database. The whole mitogenome of blue sheep was found to be highly AT-biased (60%) and had a positive AT skew (0.121) and a negative GC skew (−0.341). In 13 PCGs of blue sheep, Leucine (15.58%) and tryptophan (2.72%) occurred most frequently. A typical secondary cloverleaf structure was observed for all tRNA genes except for tRNA-Ser, where a stable structure of dihydrouridine did not develop. The phylogenetic analysis showed Indian blue sheep population formed a separate clade with a genetic distance of 3.7 to 4.1% from the Chinese blue sheep population, suggesting it to be of a different lineage and genetically qualifies the status of distinct subspecies. The results of this study will help in further phylogenetic analysis of Indian blue sheep populations in the Western and Eastern Himalayan regions and in understanding lineage identification and evolution for further research.

1. Introduction

The Himalayan blue sheep (*Pseudois nayaur*) or *bharal* is listed as a 'Least Concern' species by the International Union for Conservation of Nature (IUCN). According to the Indian Wild Life (Protection) Act, 1972, it is protected under Schedule *I*, and The Convention on International Trade in Endangered Species of Flora and Fauna lists it in Appendix III (CITES) (Harris, 2014). The species is widely distributed in India, Nepal, Bhutan, China, and northern Pakistan (Coogan et al., 2015) and are probably found in Tajikistan (Wilson and Reeder, 2005). In the Indian Himalayan Region (IHR), blue sheep are mostly found on the southern slopes from Ladakh to Arunachal Pradesh (Mallon, 1991; Uniyal, 2004; Mishra et al., 2006). The blue sheep is one of the most popular wild animals and plays a critical role in maintaining the food chain. It is primarily a grazer, but they turn to herbs and shrubs during periods of grass shortage. It is a preferred prey of snow leopards (*Panther uncia*), and Himalayan wolves (*Canis lupus*), and young are known to be predated upon by Red foxes *(Vulpes vulpes)* and eagles (Sharma and Lachungpa, 2002). The blue sheep habitat's elevation range was between 2500 and 5400 m in the Himalayan region (Aryal et al., 2016).

According to the taxonomy, the genus *Pseudois* has two species: the blue sheep (*P. nayaur*) and the dwarf blue sheep (*P. schaeferi*), two subspecies of the *P. nayaur* have been identified based on body size (Wang and Hoffmann, 1987; Bhatnagar, 2003). *P. n. nayaur* is the main subspecies found in Western Himalayas; however, Chinese blue sheep (*P. n. szechuanensis*) *is* found in the eastern Himalayan region but IUCN has not

Abbreviations: P. nayaur, *Pseudois nayaur*; *P. n. szechuanensis*, *Pseudois nayaur szechuanensis*; IUCN, International Union for Conservation of Nature; CITES, Convention on International Trade in Endangered Species of Flora and Fauna; IHR, Indian Himalayan Region; mtDNA, Mitochondrial DNA; gDNA, genomic DNA; PCR, Polymerase Chain Reaction; *Cyt b*, Cytochrome *b*; MCMC, Monte Carlo Markov Chain; NCBI, National Center for Biotechnology; PCGs, protein-coding genes; D-loop, control region; RSCU, Relative synonymous codon usage; mtCR, Mitochondrial control region; A, Adenine; G, Guanine; T, Thymine; C, Cytocine.

* Corresponding author at: Wildlife Forensic and Conservation Genetics Cell, Wildlife Institute of India, Chandrabani, Dehradun 248 001, Uttarakhand, India.
E-mail address: skg@wii.gov.in (S.K. Gupta).

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acknowledged this and hence its taxonomic status is still uncertain due to limited data. The easternmost population of *P. n. szechuanensis* are found in Helan Mountains and significantly differ from the blue sheep in Qinghai, Gansu and Sichuan Provinces of China. In India, dwarf blue sheep (*P. schaeferi*) distribution is unknown (Xiao-Ming et al., 1998; Shackleton, 1997). Several studies have been conducted to determine its current status, distribution and phylogenetics, but the blue sheep's genetic structuring and population boundaries are still unclear. Previous studies on blue sheep were centered on the mtDNA marker Cytochrome *b* (Cyt *b*), which revealed the existence of two lineages of blue sheep in the Indian Himalayan highlands. (Joshi et al., 2022). Moreover, populations of the Helan Mountains and Tibetan Plateau are geographically diverse. Based on nuclear genes, the Helan population is categorized as *P. n. szechuanensis*, while DNA analysis confirmed that the blue sheep in the Tibetan Mountains belonged to the *P. nayaur* species and the subspecies was *P. nayaur nayaur* (Gao et al., 2020). However, no in-depth information on the status or distribution of these blue sheep clades from India is available. As a result, the taxonomic classification of blue sheep populations across geographic ranges is still unclear. (). As a result, the full mitogenome may be more helpful in confirming the genetic status among different populations of blue sheep, especially for the blue sheep in India. In this study, to resolve the phylogenetic status, we sequenced the whole mitochondrial genome of blue sheep population in India and compared it to the genomes of other closely related species.

2. Materials and methods

2.1. Study area and sample collection

Nanda Devi Biosphere Reserve has a different climate due to its diverse landscape and its altitude ranging from around 1800 m to 7800 m. The reserve encompasses an ecosystem of mountains; thus, the weather is cool to cold temperatures and moderate to high rainfall. The sample has been collected from 30°85′8″N 79°45′64″E. The study area has significant fauna such as Snow leopard, Asiatic black bear (*Ursus thibetanus*), Himalayan & Kashmir musk deer (*Moschus leucogaster* and *Moschus cupreus*), and Himalayan tahr (*Hemitragus jemlahicus*), along with Blue sheep, and the prominently found vegetation types are fir, birch, rhododendron, and Juniper. Dried muscle was collected from one naturally dead animal's carcass and preserved in 70% ethanol and stored at −20° celcius in the deep freezer. DNeasy Blood & Tissue Kit was used to extract total genomic DNA (gDNA) in 100 μl. (20). The DNA quality was evaluated using a spectrophotometer and a 0.9% agarose gel dyed with ethidium bromide stain dye. The final concentration of the extracted DNA for PCR amplification was 50 ng/μl.

2.2. PCR amplification and sequencing

In this study, we have designed two sets of novel primers to amplify 8 to 9 kb size for amplicon library preparation and Illumina sequencing to cover the entire mitogenome of blue sheep. The amplification length for the first primer was 9014 at an annealing temp of 58 °C: BlueSheepF-1 5'-ACAACCCACGAGCCACAGAAGC-3′ and BlueSheepR1 5'-GGAATCGGGGTTGTCCTAGGAGTG-3′. The second primer amplifies 8596 bp at an annealing temp of 59 °C: BlueSheepF2 5'-TTCGGCCTACACCCATGACTACCC-3′ and BlueSheepR2 5'-GCCTGAGGATAGGGGAATGCCTTG-3′. The primers were amplified using a long-range PCR kit (TaKaRa LA Taq® DNA Polymerase, cat # RR002M). With 2 μl of 10× LA PCR Buffer ll (Mg2+ plus) and 4 μl of dNTP Mixture (2.5 mM each), 3 pmol of each primer, and 100 ng template DNA, PCR reactions were carried out in 20 μl reaction volumes. There were negative controls present for each reaction. The following conditions were used to conduct the PCR: 30 cycles at 94 °C for one minute, at 98 °C for 10 s, and 68 °C for 15 min, with a final extension of 72 °C for 10 min. Positive controls were used to assess the efficiency and uniformity of the PCR reactions. The 1% agarose gel dyed with green stain dye allowed the UV-visualization of the amplified PCR amplicons.

2.3. Library preparation and sequencing

The amplified PCR products were used for a paired-end library with an insert size of 309 bp (24.7 ng/ul concentration) was constructed using NEBNext® Ultra™ II FS DNA Library Prep Kit (Biolabs, N.E., 2016. NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®) as per manufacturer's protocols. The quality and quantity of the prepared library were assessed using Agilent 4150 TapeStation system and Qubit 4 Fluorometer. High-quality DNA library was sequenced (2 × 150 bp) on NovaSeq 6000 V1.5.

2.4. Blue sheep mitogenome raw data statistics, assembly, and characterization

A total of 9,125,572 raw reads of mitochondrial genome sequences were quality-checked through fastqc tool kit followed by removing the adaptor using '*fastp*' in default settings. The filtered reads were further used for the *K-mer* optimization and seed size selection using the 'GetOrganelle' program. Finally, the assembly was carried out through the 'spades assembler' to get the circular mitochondrial organelle genome of 16,718 in size. For the comparative study, we used full mtDNA sequences from the Blue sheep species and subspecies (JX101653 (Halen Mountain_China), NC 020632 (Zoo, Jardin des Plantes), KP998469 (Kangding County, Sichuan Province in China), JX101652 (Tibetan Plateau) from the GenBank. The base composition and mtDNA genetic code were calculated using MEGA X. Skew analysis was used to calculate the nucleotide composition bias over the whole mitogenome, where AT skew = (A T)/(A+ T) and GC skew = (G C)/(G + C). MEGA X was applied to estimate the mitochondrial protein-coding genes' (PCGs) relative synonymous codon use (RSCU) and amino acid composition. tRNAscan-SE v2.0 was used to predict the usual Transfer RNA genes that have a secondary cloverleaf shape (tRNAs).

2.5. Phylogenetic analysis and genetic differentiation

The Tamura-Nei model (TN93) was applied to MEGA X to determine the mean pairwise genetic distance between blue sheep (Kumar et al., 2018). A total of 17 full mitogenome sequences were KT345703, FJ207536, NC0091941, NC026063, KP998469, KJ784494, FJ207537, NC020632, JX101653, JX101652, KR059225, FJ207526, MK748332, NC006295, NC024818, NC020614, NC012095 from the NCBI GenBank, including sequences from the Caprini, Bovini, and Boselaphini families and one sequence from the outgroup *Sus scrofa*, were included to analyse the phylogenetic relationships of blue sheep. BEAST v1.7 was used to generate a Monte Carlo Markov Chain (MCMC) based Bayesian consensus tree (Drummond et al., 2012). We used MCMC chains to conduct a Bayesian inference analysis for 10 million generations, sampling a tree every 1000 generations with a burn-in of 5000 generations. FigTree v1.4.0 was used to depict the phylogenetic tree (Rambaut, 2012).

3. Results

3.1. Mitogenome topology

Illumina sequencing generated the complete mitogenome of blue sheep, which was submitted to the NCBI GenBank (OP583799) (Fig. 1). There were two ribosomal RNA genes, 22 transfer RNA genes, 13 protein-coding genes (PCGs), and a non-coding control region (D-loop region) in it (Fig. 1 and Table 1). The nucleotide ratio of the mtDNA from blue sheep was A (33.6%), T (26.3%), C (26.9%), and G (13.2%). (Table 2). Except for the eight tRNA genes, the majority of the genes were encoded on the H-strand (tRNAGln, tRNAAla, tRNAAsn, tRNACys, tRNATyr, tRNASer, tRNAGlu, tRNAPro). The control region was

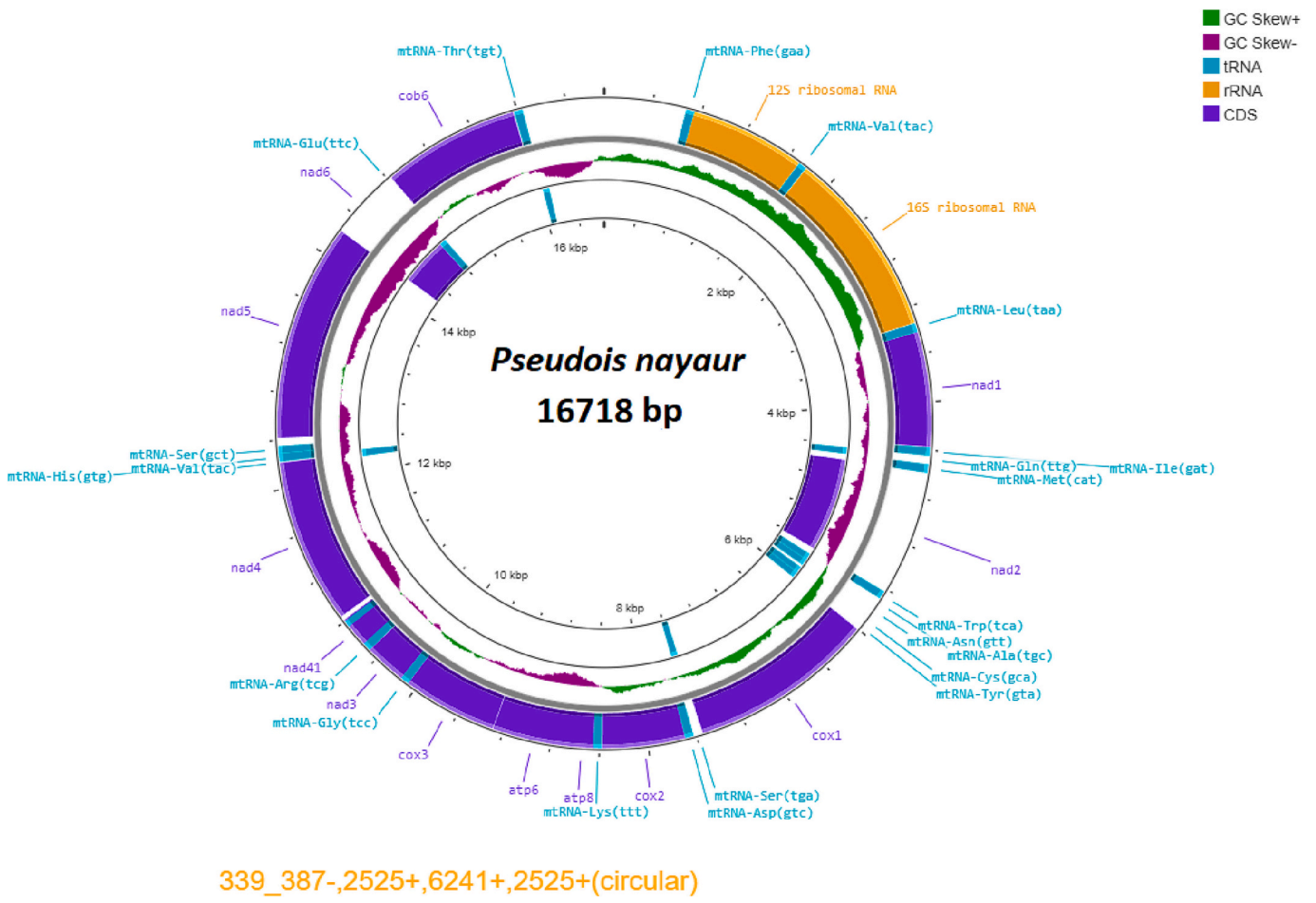

Fig. 1. Illustration of the location of genes on the complete mitochondrial genome of *Pseudois nayaur* (16,718 bp).

between the tRNAPro and the tRNAPhe (Table 1). We identified eight pairs of overlapping genes among the tRNAIle/tRNAGln, COI/tRNASer, ATP8/ATP6, ATP6/COIII, ND4L/ND4, ND5/ND6, and tRNAThr/tRNAPro. The overlapping was lowest (1 bp) between ATP6/COIII and tRNA-Thr/tRNA-Pro, and largest (40 bp) between ATP8 and ATP6 (Table 1). A total of 13 intergenic spacers between the mitochondrial regions were found, ranging in size from 1 to 32 bp. The largest gap (32 bp) was observed between tRNAAsn and tRNACys (Table 1). The whole mitogenome of blue sheep was observed to have an AT bias of 60% and 40% of the AT and GC content. We computed the values of AT-skew, GC-skew, AT.%, and GC% for characterizing nucleotide compositions in the whole mitogenome. All the studied blue sheep species had positive AT-skew (0.112), while the remaining had negative GC-skew (−0.341).

3.2. Protein-coding genes (PCGs)

With a total length of 11,319 bp and 64 bp overlapping fragments, the 13 PCGs in the blue sheep mitogenome comprised 67.70% of the entire mitogenome. PCGs typically have the following base compositions: A = 31.4%, *T* = 27.7%, G = 13.0%, and C = 27.9%. (Table 2). PCGs typically have a base composition of A = 31.4%. G is 13.0%, T is 27.7%, and C is 27.9%. (Table 2). The cytochrome *c* oxidases CO*I*, CO*II*, and CO*III*, the NADH dehydrogenases ND1, ND2, ND3, ND4, ND5, and ND4L, the ATPases ATP6 and ATP8, the cytochrome *b* gene: Cyt *b* gene and the minority strand or L-strand gene (NADH dehydrogenase: ND6 gene) are among the 12 majority strand or H-strand (Fig. 1 and Table 1). AT.% was more frequent than GC% (59.1% vs. 40.9%). To elucidate the nucleotide distribution in PCGs, we evaluated the base skews between different blue sheep species. For blue sheep PCGs, the average AT and GC skews values were 0.062 and 0.364, respectively. The nucleotide composition was skewed by C, as indicated by the negative GC skewness values. The ND5 gene (1821 bp) was the longest, and the ATP8 gene (201 bp) was the shortest among the 13 PCGs. ATG or ATA was used to start all 13 PCGs. Out of the 13 PCGs, we observed seven complete stop codons (TAA), whereas ND1- ND4 and COIII used incomplete codons (TA- or T-) (Table 1). A post-transcriptional polyadenylation addition occurred throughout the mRNA maturation process to complete the PCGs that lacked a complete stop codon. The 13 PCGs of blue sheep have a relative synonymous codon use (RSCU) of 3773 codons (stop codons excluded) (Fig. 2). Leucine (15.58%) and tryptophan (2.72%) were found in blue sheep PCGs at the greatest and lowest frequencies, respectively (Fig. 3).

3.3. Ribosomal RNA and transfer RNA genes

The entire blue sheep mitogenome was found to have 22 tRNA genes and two ribosomal RNA genes. 12S rRNA was 957 bp in size, whereas 16S rRNA was 1573 bp (Table 1 and Fig. 1). Two rRNA included the following nucleotide percentages: A (36.9%), T (23.4%), C (22.1%), and G (17.7%). (Table 2). Blue sheep's two rRNA genes were 2531 base pairs long, making up 15.01% of the whole mitogenome. Two rRNA had a total AT content of 61.30%, and the AT and GC skew were, respectively, 0.022 and − 0.110. The 22 tRNA genes were dispersed over the whole mitogenome, and their sizes ranged from 60 base pairs (tRNAser) to 75 base pairs (tRNALeu). Of these 22 tRNA genes, eight were on the L-strand and 14 were on the H-strand (Fig. 1 and Table 1). The nucleotide composition of 22 tRNA was A (32.9%), T (31.0%), C (17.0%), and G (19.0%) and its size was 1512 bp. It was observed that tRNA had an AT

Table 1
Organization of the complete mitochondrial genome of *Pseudois nayaur*.

	Positions			Codon				
	Start	End	Size	Start	Stop*	Anti-codon	Strand	Space/overlap#
tRNA-Phe	1	68	68	–	–	GAA	H	0
12S ribosomal RNA	69	1025	957	–	–	–	H	0
tRNA-Val	1026	1092	67	–	–	TAC	H	0
16S ribosomal RNA	1093	2665	1573	–	–	–	H	0
tRNA-Leu	2666	2740	75	–	–	TAA	H	0
ND1	2743	3698	956	ATG	TA–	–	H	+2
tRNA-Ile	3699	3767	69	–	–	GAT	H	0
tRNA-Gln	3765	3836	72	–	–	TTG	L	–3
tRNA-Met	3839	3907	69	–	–	CAT	H	+2
ND2	3908	4949	1042	ATA	T–	–	H	0
tRNA-Trp	4950	5016	67	–	–	TCA	H	0
tRNA-Ala	5018	5086	69	–	–	TGC	L	+1
tRNA-Asn	5088	5160	73	–	–	GTT	L	+1
tRNA-Cys	5193	5260	68	–	–	GCA	L	+32
tRNA-Tyr	5261	5328	68	–	–	GTA	L	0
COI	5330	6874	1545	ATG	TAA	–	H	+1
tRNA-Ser	6872	6942	71	–	–	TGA	L	–3
tRNA-Asp	6948	7015	68	–	–	GTC	H	+5
COII	7017	7700	684	ATG	TAA	–	H	+1
tRNA-Lys	7704	7771	68	–	–	TTT	H	+3
ATP8	7773	7973	201	ATG	TAA	–	H	+1
ATP6	7934	8614	681	ATG	TAA	–	H	−40
COIII	8614	9397	784	ATG	T–	–	H	−1
tRNA-Gly	9398	9466	69	–	–	TCC	H	0
ND3	9467	9812	346	ATA	T–	–	H	0
tRNA-Arg	9813	9882	70	–	–	TCG	H	0
ND4L	9883	10,179	297	ATG	TAA	–	H	0
ND4	10,173	11,550	1378	ATG	T–	–	H	−7
tRNA-His	11,551	11,619	69	–	–	GTG	H	0
tRNA-Ser	11,620	11,679	60	–	–	GCT	H	0
tRNA-Leu	11,681	11,750	70	–	–	TAG	H	+1
ND5	11,751	13,571	1821	ATA	TAA	–	H	0
ND6	13,555	14,082	528	ATG	TAA	–	H	−17
tRNA-Glu	14,083	14,151	69	–	–	TTC	L	0
CYTB	14,156	15,295	1140	ATG	AGA	–	H	+4
tRNA-Thr	15,299	15,368	70	–	–	TGT	H	+3
tRNA-Pro	15,368	15,433	66	–	–	TGG	L	−1
Control region	15,434	16,718	1285	–	–	–	H	0

Table 2
Nucleotide composition and skewness in the *P. nayaur* mitochondrial genome.

P.nayaur	Size(bp)	A%	T%	AT-skew	G%	C%	GC-skew
whole Mitogenome	16,718	33.6%	26.3%	0.121	13.2%	26.9%	−0.341
PCGs	11,319	31.4%	27.7%	0.062	13.0%	27.9%	−0.364
tRNAs	1512	32.9%	31.0%	0.029	19.0%	17.0%	0.055
rRNAs	2531	36.9%	23.4%	0.022	17.7%	22.1%	−0.110
Control region	1285	37.8%	24.2%	0.211	12.3%	26.0%	−0.436

bias, with an overall AT and GC content of 63.9% and 36.0%, respectively. Positive skews values were found for the AT content (0.029) and the GC content (0.055). (Table 2). Table 1 lists the anti-codons of 22 tRNAs from blue sheep. Except for tRNAser, it did not create a stable structure with its dihydrouridine arm, and all 21 tRNA genes displayed a common secondary cloverleaf pattern (Figs. 4 and 5).

3.4. Mitochondrial D-loop

In *P. nayaur*, the mitochondrial control region/D loop(mtCR) was 1285 bp in length and was located between the tRNAPro and tRNAPhe (Table 1 and Fig. 1). Due to insertion and deletion, the CR length varies (INDEL). A (37.8%), T (24.2%), C (26.0%), and G (12.3%) were the nucleotide components of CR. The AT content (62%) was greater than the GC content (38.3%). AT has positive skews values, whereas GC content has negative skews values were found to be 0.211 and − 0.436, respectively (Table 2).

3.5. Phylogenetic analysis and genetic distance

Complete mitogenome sequences of Chinese blue sheep, seven Caprini, one Bovini, and two species of Boselaphini were used to determine the evolutionary position of blue sheep. The Bayesian inference phylogenetic tree showed that all blue sheep species were unique from other Cervini, Bovini, and Boselaphini species and clustered in a monophyletic clade (Fig. 6). Except for dwarf blue sheep (PP = 0.97), the phylogenetic tree topology derived from Chinese and Indian blue sheep populations had high posterior probability values (PP = 1). The phylogenetic study revealed that the Indian blue sheep population represented a distinct group. Using the whole mitogenome, we calculated the pairwise genetic distance between the species (Table 3). The highest genetic distance was found between the blue sheep clade from India and China (JX101653), with a genetic distance of (0.041, 4.1%) followed by *P. n. szechuanensis* (KP998469, 3.9%), and *P. n. nayaur* (JX101652, 3.7%). However, a low genetic distance (1.9%) was observed between *P. n. szechuanensis* (KP998469) and *P. nayaur*

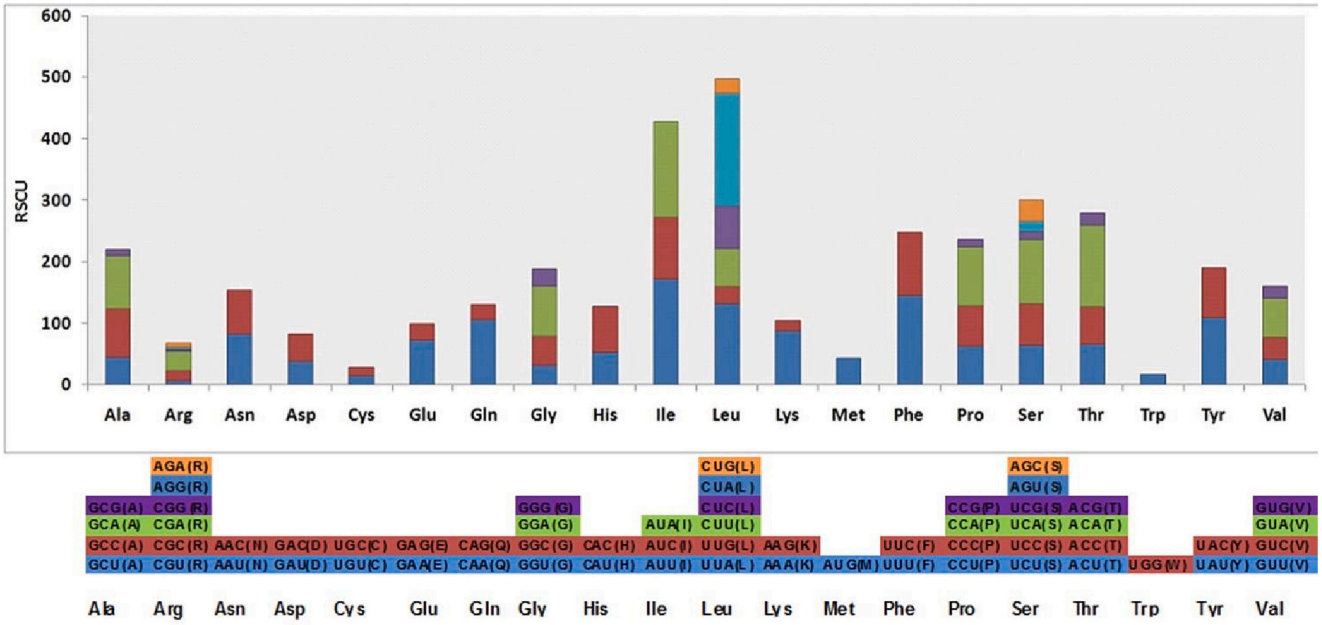

Fig. 2. Relative synonymous codon usage (RSCU) of the mitochondrial protein-coding genes *Pseudois nayaur* of the mitochondrial genome. Codon count numbers are provided on the X-axis.

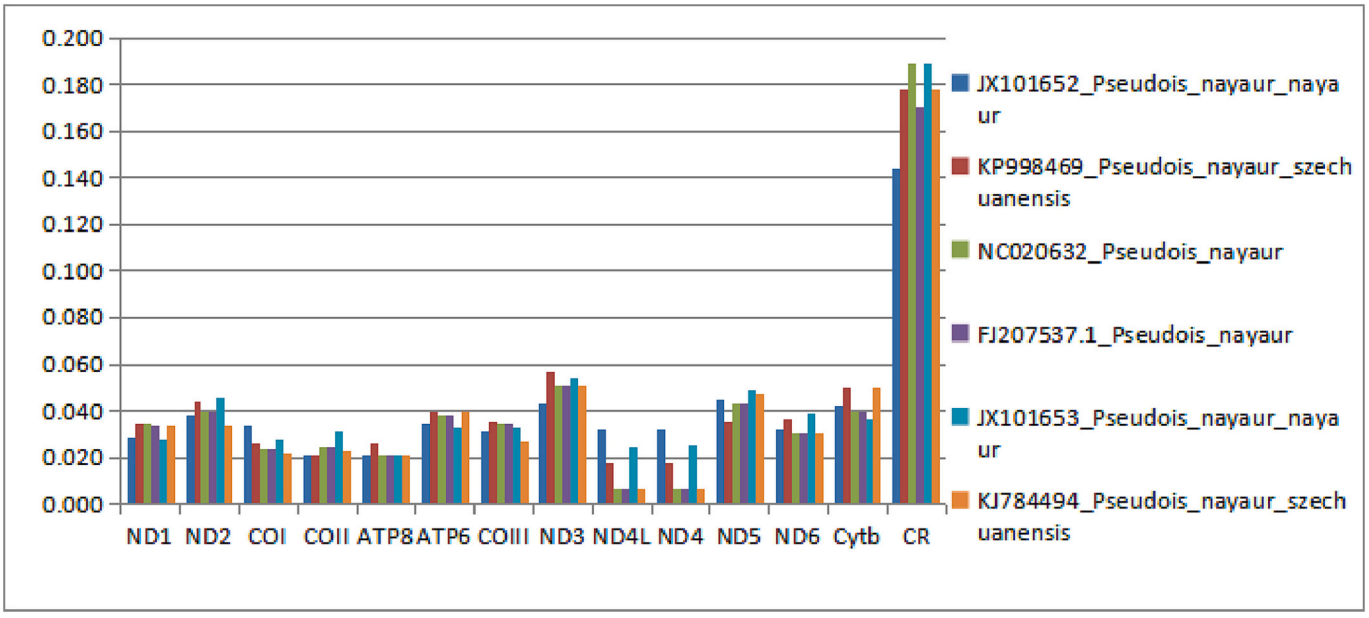

Fig. 3. Comparative pairwise genetic distance in the protein-coding genes and control region of other subspecies of blue sheep from *Pseudois nayaur*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(NC020632). It indicated that the newly generated complete mitogenome of the Indian blue sheep population was a different lineage, genetically qualifying the status of distinct subspecies. The gene-wise comparison of the species and subspecies of blue sheep is shown in Fig. 3.

4. Discussion

The complete mitogenome sequence of blue sheep from the Indian Himalayas was unavailable in the open database. Using the Illumina Nova-seq 6000 technology, we sequenced the first whole mitogenome of blue sheep (16,718 bp) from Nanda Devi Biosphere Reserve in Uttarakhand, India. The arrangement of genes was comparable to that of other mammalian species (Kumar et al., 2018; Singh et al., 2020). In the complete mitogenome of blue sheep, we found eight pairs of overlapping genes, similar to other deer species (Gilbert et al., 2006). We discovered that *Pseudois nayaur*'s mtCR was 1285 bp long and positioned between the tRNAPro and tRNAPhe. The mitochondrial genome's replication and transcription are significantly regulated by the mtCR, a non-coding, hyper-variable region (Pesole et al., 1999). In IHR, blue sheep are mostly found on the southern slopes from Ladakh (Pamir plateau) to Arunachal Pradesh (Tibetan plateau). Recently, five haplotypes in blue sheep populations were identified using a short fragment of Cyt *b* (Joshi et al., 2022). Western and Eastern blue sheep populations were found in the Tibetan and Pamir plateaus (Joshi et al., 2022). Both of these were genetically diverged and paraphyletic (Tan et al., 2017). A comparison

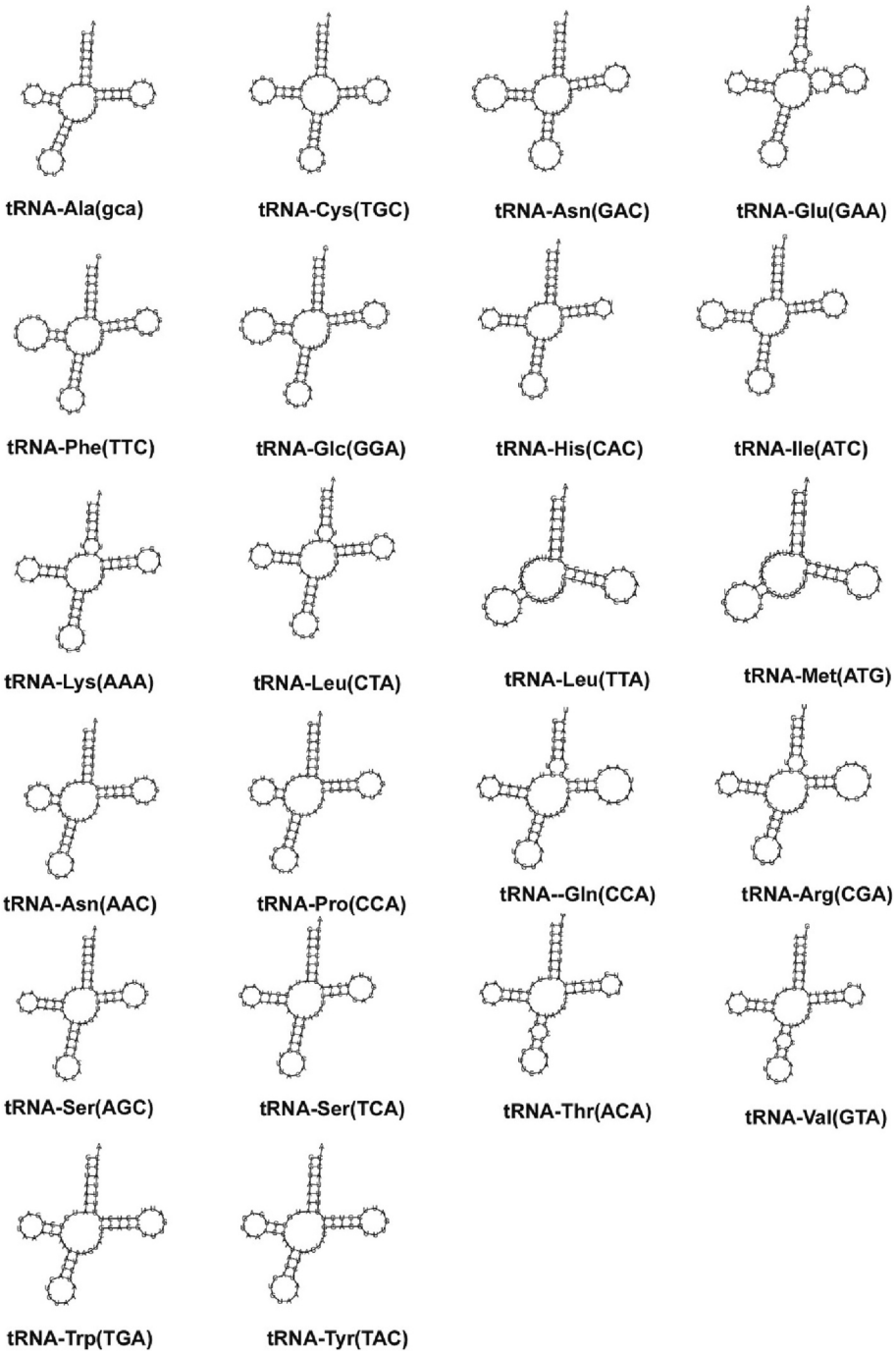

Fig. 4. Predicted secondary structures of the 22 tRNA genes in *Pseudois nayaur*.

of the newly generated blue sheep sequence with GenBank sequences from the Chinese indicated a separate clade from India with a genetic distance ranging from 3.7 to 4.1%. However, a short Cyt *b* sequence-based study showed its clustering within the Western Himalaya-Pamir plateau (Tan et al., 2017).

In contrast to the small segment, increased mtDNA coverage provided more information about the phylogenetic tree's resolution and taxonomic position (Boore, 1999). The control region was highly variable, in which *P. nayaur* (JX101653) showed a high genetic distance from the Indian sample. We observed that the Indian blue sheep had the longest (1285 bp), and *P. n. szechuanensis* (KJ784494) had the lowest (1084 bp) mtCR. Our research also suggested that a complex interplay of environmental and historical factors has shaped the unique evolutionary history of blue sheep populations in the Himalayan region.

5. Conclusions

The complete mitogenome blue sheep, which was 16,718 base pairs long, was sequenced and characterized. The phylogenetic analysis revealed that Indian blue sheep formed a separate clade with a genetic distance of 3.7 to 4.1% from the Chinese blue sheep population and suggested it is a different lineage, which genetically qualifies the status of distinct subspecies. The fundamental information needed for phylogenetic analysis, geographical distribution ranges, and evolutionary connections was derived from a comparative analysis of blue sheep species based on the entire mitogenome. Longer mtDNA sequencing is

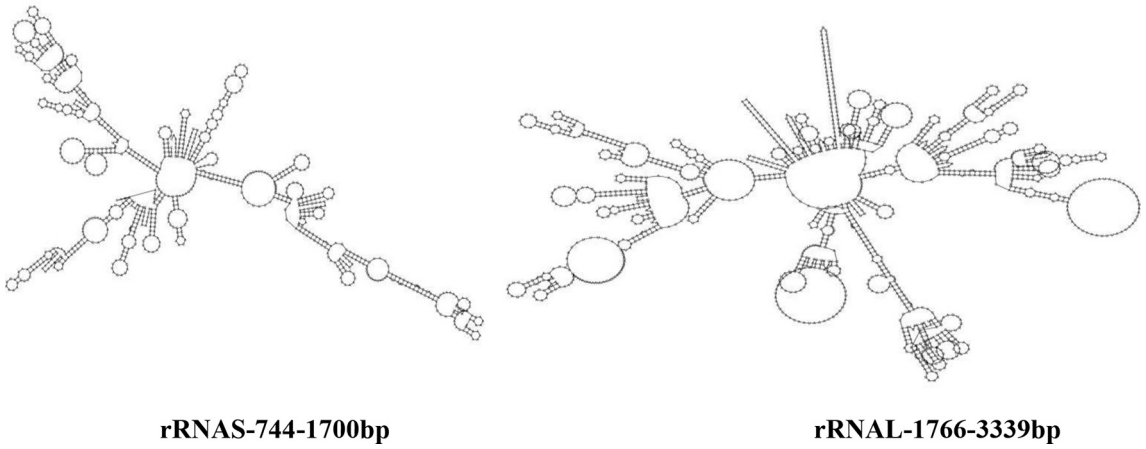

Fig. 5. Predicted secondary structures of the rRNA genes in *Pseudois nayaur*.

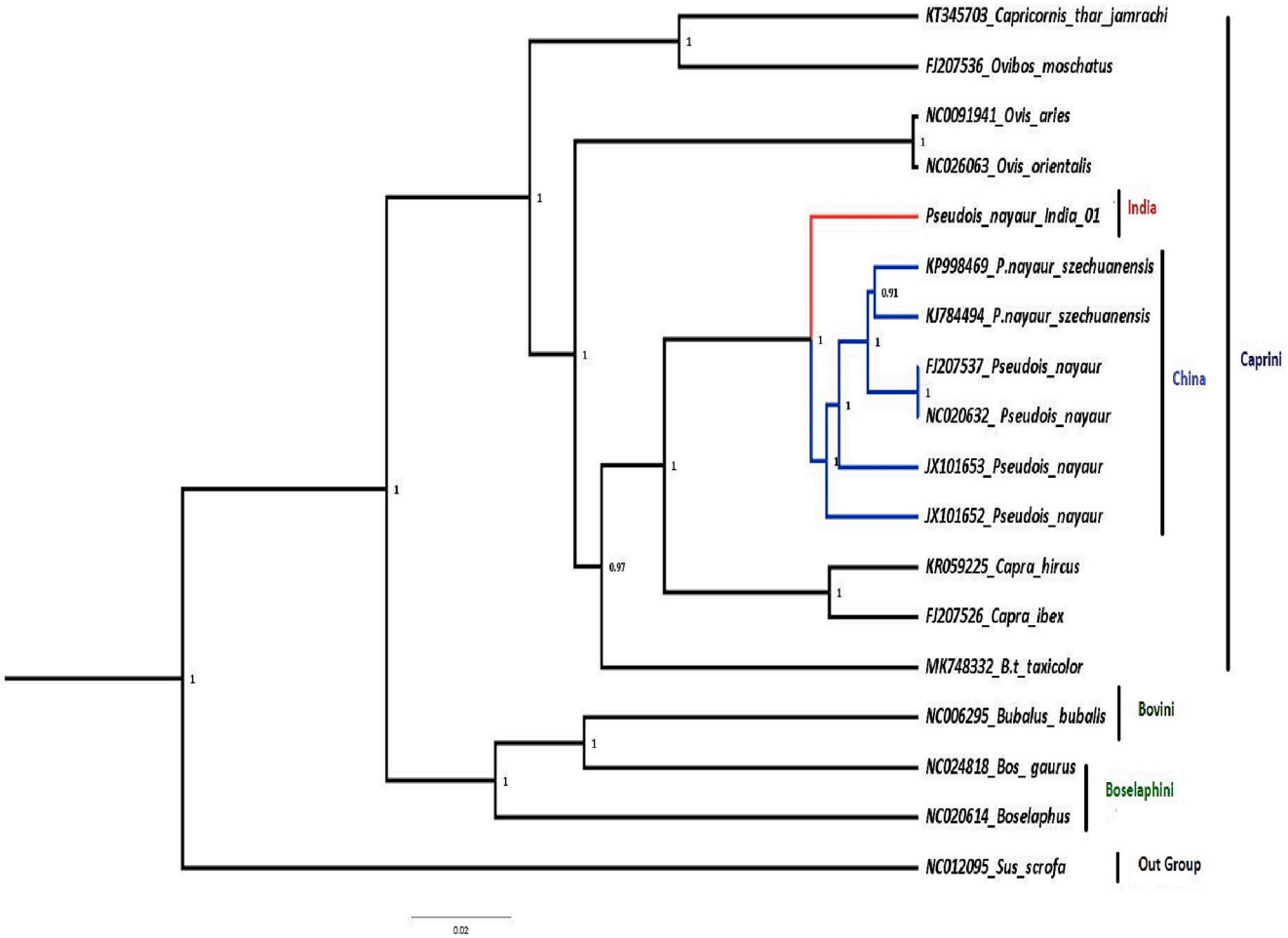

Fig. 6. Phylogenetic relationship of *Pseudois nayaur* with other species of blue sheep based on Complete mitogenome. Bayesian posterior probability values are shown at each node. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

helpful in determining whether a group of populations harboring a high amount of genetic diversity could justify separate conservation efforts. The newly sequenced blue sheep mitogenome (from India) will also help with the lineage identification of the biological materials seized for investigating wildlife crime cases. It provided baseline data for deeper insight into the phylogenetic analysis of other Indian blue sheep populations from the Western Himalayan region (Himachal Pradesh and Ladakh) and Eastern Himalayan region (Sikkim and Arunachal Pradesh). By understanding these factors, conservationists and wildlife managers can develop more effective strategies for protecting these important species and their habitats in the face of ongoing environmental challenges.

Declaration of Competing Interest

The authors declare no competing interests.

Table 3
Genetic differentiation among Blue sheep species of *Pseudois nayaur* are presented (below the diagonal) and their standard errors (above the diagonal).

	Species	1	2	3	4	5	6
1	Mitochondrial_genome_India_1		0.002	0.002	0.002	0.002	0.002
2	JX101653.1_*Pseudois_nayaur*	0.041		0.002	0.002	0.002	0.002
3	NC_020632.1_*Pseudois_nayaur*	0.038	0.033		0.001	0.001	0.002
4	KP998469.1_*Pseudois_nayaur_szechuanensis*	0.039	0.035	0.019		0.001	0.002
5	KJ784494.1_*Pseudois_nayaur_szechuanensis*	0.038	0.033	0.019	0.020		0.002
6	JX101652.1_*Pseudois_nayaur_nayaur*	0.037	0.036	0.034	0.035	0.034	

Data availability

Data freely available at NCBI GenBank

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References

Aryal, A., Shrestha, U.B., Ji, W., Ale, S.B., Shrestha, S., Ingty, T., Maraseni, T., Cockfield, G., Raubenheimer, D., 2016. Predicting the distributions of predator (snow leopard) and prey (blue sheep) under climate change in the Himalaya. Ecol. Evol. 6 (12), 4065–4075.

Bhatnagar, Y.V., 2003. Species of the Trans-Himalaya and other arid tracts. In: Sathyakumar, S., Bhatnagar, Y.V. (Eds.), Mountain Ungulates. ENVIS Bulletin: Wildlife and Protected Areas, pp. 44–49.

Boore, J.L., 1999. Animal mitochondrial genomes. Nucleic Acids Res. 27 (8), 1767–1780.

Coogan, S.C., Ji, W., Rothman, J.M., Raubenheimer, D., 2015. Foods, macronutrients and fibre in the diet of blue sheep (*Psuedois nayaur*) in the Annapurna conservation area of Nepal. Ecol. Evol. 5 (18), 4006–4017.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29 (8), 1969–1973.

Gao, H., Li, N., Huang, Y., Qiao, F., Li, J., Li, Z., Li, Y., Wang, Z., Teng, L., Liu, Z., 2020. Taxonomic status of Chinese blue sheep (Pseudois nayaur): new evidence of a distinct subspecies. Integ. Zool. 15 (3), 202–212.

Gilbert, C., Ropiquet, A., Hassanin, A., 2006. Mitochondrial and nuclear phylogenies of Cervidae (Mammalia, Ruminantia): systematics, morphology, and biogeography. Mol. Phylogenet. Evol. 40 (1), 101–117.

Harris, R.B., 2014. *Pseudois nayaur*. The IUCN Red List of Threatened Species 2014:e. T61513537A64313015. https://doi.org/10.2305/IUCN.UK.2014-3.RLTS.T61513537A64313015.en. Accessed on 19 May 2023.

Joshi, B.D., Kumar, V., De, R., Sharma, R., Bhattacharya, A., Dolker, S., Pal, R., Kumar, V. P., Sathyakumar, S., Adhikari, B.S., Habib, B., 2022. Mitochondrial cytochrome b indicates the presence of two paraphyletic diverged lineages of the blue sheep Pseudois nayaur across the Indian Himalaya: conservation implications. Mol. Biol. Rep. 1–10.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35 (6), 1547.

Mallon, D.P., 1991. Status and conservation of large mammals in Ladakh. Biol. Conserv. 56 (1), 101–119.

Mishra, C., Madhusudan, M.D., Datta, A., 2006. Mammals of the high altitudes of western Arunachal Pradesh, eastern Himalaya: an assessment of threats and conservation needs. Oryx 40 (1), 29–35.

Pesole, G., Gissi, C., De Chirico, A., Saccone, C., 1999. Nucleotide substitution rate of mammalian mitochondrial genomes. J. Mol. Evol. 48 (4), 427–434.

Rambaut, A., 2012. A Graphical Viewer of Phylogenetic Trees, FigTree v1. 4.0. See. http://tree.bio.ed.ac.uk/software.figtreetree.

Shackleton, D.M., 1997. Wild Sheep and Goats and their Relatives: Status Survey and Conservation Action Plan for Caprinae.

Sharma, T.R., Lachungpa, U., 2002. Status, distribution and management of mountain ungulates in Sikkim. (IN) Sathyakumar, S and Bhatnagar, Y.V (Eds.) mountain ungulates. ENVIS Bull.: Wildlife Protect. Areas Wildlife Inst. India 1 (1), 38–43.

Singh, B., Gautam, K.B., Sahoo, S., Kumar, A., Gupta, S.K., 2020. Complete mitogenome of Kashmir musk deer (Moschus cupreus) and its comparative phylogenetic relationships. Mol. Biol. Rep. 47 (12), 9757–9763.

Tan, S., Wang, Z., Jiang, L., Peng, R., Zhang, T., Peng, Q., Zou, F., 2017. Molecular phylogeny and phylogeography of genus Pseudois (Bovidae, Cetartiodactyla): new insights into the contrasting phylogeographic structure. Ecol. Evol. 7 (17), 7047–7057.

Uniyal, V.P., 2004. Bharal-the most sighted ungulate of Nanda Devi National Park. Tiger Pap. 31 (1), 21–24.

Wang, X., Hoffmann, R.S., 1987. Pseudois nayaur and Pseudois schaeferi. Mamm. Species 278, 1–6.

Wilson, D.E., Reeder, D.M., 2005. Mammal Species of the World: A Taxonomic and Geographic Reference, vol. 1. JHU Press.

Xiao-Ming, W.A.N.G., Zhi-Xiao, L.I.U., Hong-Fa, X.U., Ming, L.I., Yuan-Guang, L.I., 1998. The blue sheep population ecology and its conservation in Helan Mountain, China. Biodivers. Sci. 6 (1), 1.

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ORIGINAL ARTICLE

Molecular Taxonomy Suggests Presence of Two Distinct Lineages of Blue Sheep (*Pseudois nayaur*) in Indian Himalayan Region

Deepesh Saini[1] · Gaurav Sonker[1] · Tushar Parab[1] · Vishnupriya Kolipakam[1] · Sambandam Sathyakumar[1] · Salvador Lyngdoh[1] · Sandeep Kumar Gupta[1]

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Abstract

Indian Himalayan Region (IHR) supports a plethora of biodiversity with a unique assemblage of many charismatic and endemic species. We assessed the genetic diversity, demographic history, and habitat suitability of blue sheep (*Pseudois nayaur*) in the IHR through the analysis of the mitochondrial DNA (mtDNA) control region (CR) and Cytochrome *b* gene, and 14 ecological predictor variables. We observed high genetic divergence and designated them into two genetic lineage groups, i.e., the Himalayan blue sheep (*P. n. nayaur*) in the western part, and the Chinese blue sheep (*P. n. szechuanensis*) in the eastern part. They exhibited poor connectivity due to landscape resistance. The genetic distance value suggested substantial genetic differentiation between them. The habitat selection by blue sheep indicated the disparity between the residence preferences in the western and eastern Himalayas. In both the regions, the habitat suitability was mostly influenced by the minimum temperature of the coldest month. However, in the eastern Himalayas, precipitation seasonality emerged as a significant variable influencing habitat suitability. These findings provided strong support for the presumption that the habitats preferred by blue sheep in the western Himalayas are dryer, compared with the preferred habitats in the eastern region, which were moister. The identification of two separate lineages of *P. nayaur* in the IHR has significant conservation implications as it underlines the necessity for a unique management approach for each lineage. In order to preserve genetic integrity, conservation efforts must make sure that each population is maintained and monitored separately, as genetic divergence across the lineages that might indicate reproductive isolation. This study has potential conservation implications as it provides insights on the crucial ecological information of a relatively lesser-known ungulates species of Himalaya essential for effective conservation planning.

Extended author information available on the last page of the article

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Keywords Mitochondrial DNA (mtDNA) · Control region (CR) · Cytochrome b (Cyt b) gene · Chinese blue sheep · Lesser-known ungulates

Introduction

The in-depth understanding of a species' genetic status and ecological requirements plays a crucial role in ensuring the long-term persistence of a species (Moritz 1994) particularly in the alpine region where the free-ranging terrestrial wildlife are polytypic due to low permeability and connectivity between different habitats (Benton and Bowler 2012; Dolker et al. 2023; Bhattacharya et al. 2020; Dolker et al. 2023). Therefore, assessing genetic distance among the populations and studying the impact of habitat connectivity throughout the distribution range are essential to interpret the evolutionary consequences in mountainous ranges (Bhattacharya et al. 2020; Dolker et al. 2023). In this context, mitochondrial DNA (mtDNA) data is used along with direct and indirect sign surveys to address questions on phylogeography, evolutionary history, current population genetic parameters, and species distribution with respect to landscape features and habitat connectivity. This empirical information has significant conservation implications as it helps prioritize the conservation and management goals for the species in the distributional range, and eventually, conserve suitable habitats for the population (Manel et al. 2003; Dalui et al. 2020; Joshi et al. 2020; Ghosh et al. 2022; Dolker et al. 2023).

Blue sheep (*Psudois nayaur*) is one of the most commonly found wild ungulates in the region of the Tibetan plateau, Pamir plateau, and Indian Himalayan Region (IHR) distributed in China, Bhutan, India, Myanmar, Nepal, Pakistan, and Tajikistan (Tan et al. 2012). Based on their geographic origin and genetic variation, it was suggested that blue sheep should be classified into different subspecies (Wang et al. 2006). The Helan mountain population in China was proposed as a new subspecies, *P. n. helanshanensis* owing to its distinct morphological and genetic characteristics (Wang et al. 2006). Another ambiguous taxonomic issue was the status of the dwarf blue sheep (*P. schaeferi*) found only in the Sichuan province of China (Wang et al. 2000; Yu and Xiao-Ming 2003; Zeng et al. 2008Tan et al. 2012a). However, few studies demonstrated that dwarf blue sheep are not a separate species but a subset of *P. n. nayaur*, the eastern Himalayan subspecies of blue sheep, and *P. n. szechuanensis* from the Sichuan subspecies, which is recognized in the phylogenetic classification (Wang et al. 2006; Tan et al. 2017; Saini et al. 2023; Dolker et al. 2023).

Blue sheep is a Schedule I species under the Wildlife (Protection) Act, 1972 and listed as least concerned (LC) in the IUCN Red List, primarily found in the western Tawang region of Arunachal Pradesh, Himachal Pradesh, Uttarakhand, Sikkim, and Ladakh at altitudes between 2500 m and 5500 m above the sea level (a.s.l.) (Schaller 1998; Namgail et al. 2009; Harris 2014; Saini et al. 2023). However, from the western and eastern Himalayas, the topography acts as a barrier for the east to west movement of the species inside their distributional range. They exhibit an altitudinal migration to lower elevations during winter to meet their nutritional requirement (Bhatnagar et al. 2019). Climate change, increasing anthropogenic pressure and livestock grazing practices are the major

factors negatively affecting the species distribution range and also their population (Farooquee and Nautiyal 1999; Mishra et al. 2004; Namgail et al. 2007; Suryawanshi 2008; Bhattacharya et al. 2020).

We conducted this study to assess the genetics distance and preferred habitat of blue sheep in the western and eastern parts of the IHR using mtDNA control region (CR) and Cytochrome *b* (cyt *b*) gene along with ecological modeling. We tested whether the populations of blue sheep in the eastern and western Himalayas belong to different lineages and have different habitat preferences. Eastern Himalayas blue sheep are considered as a subset of Tibetan Plateau blue sheep, whereas the western Himalayan species are the subset of Pamir Plateau blue sheep.

Materials and Methods

Study Region and Sample Collection

35 fecal samples were collected from the western Himalayas (Union Territory of Ladakh, Uttarakhand, and Himachal Pradesh) and five from eastern Himalayas (Sikkim) (Fig. 1) during July 2022 to July 2023 with appropriate permissions. Samples were stored in 70% ethanol until DNA extraction. Total genomic DNA (gDNA) was

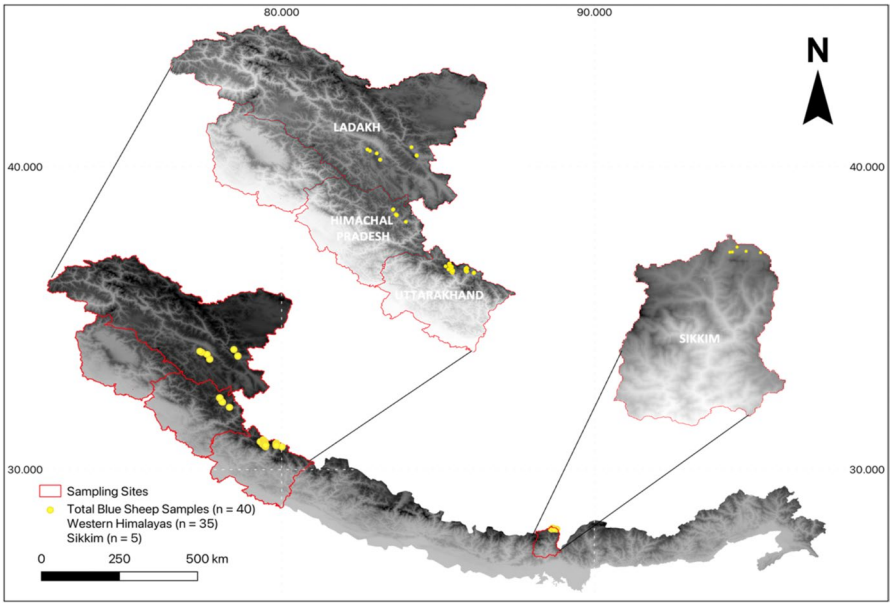

Fig. 1 A map illustrating the distribution and sampling locations for the current study. Yellow dots indicate the location of all the sampling sites (This map was created in-house using the template map of the Survey of India)

extracted in 100 µl volume using the GuHCl method (Gupta et al. 2013) and DNeasy Blood & Tissue Kit. The quality of DNA was checked under UV light on 0.8% agarose gel stained with EtBr and quantified using a QIAxpert spectrophotometer.

PCR Amplification and Sequencing

PCR amplification and DNA sequencing were carried out for CR using newly designed primer set: Forward 5′-CTATAGCCTCACTATCAGCAC-3′ and Reverse: 5′- AGCACAGTT ATGTGTGAGC-3′ and for cyt *b* gene using mcb398 5'-TAC CATGAGGACAAATATCATT CTG-3′ and mcb869 5′-CCTCCTAGTTTGTTA GGGATTGATCG-3′ (Verma & Singh 2003). The PCR reactions were performed in 10 µl reaction volumes using 1 × PCR buffer (50 mM KCl, 10 mM Tris–HCl and pH 8.0), 0.25 mM of each dNTPs, 1.5 mM $MgCl_2$, 3 pmol of each primer, 0.6 units of DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) and 4 µl of the template DNA. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, then 38 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1:30 s, followed by a final extension at 72 °C for 10 min. A negative and positive control were used to ensure the experiment's reliability. PCR amplification was confirmed by electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 mg/ml) and viewed with a UV transilluminator. The successfully amplified PCR products were treated with alkaline phosphatase and exonuclease-*I* at 37 °C for 20 min to remove any remaining primer and dNTPs, followed by 15 min of enzyme deactivation at 85 °C for subsequent Sanger sequencing. The purified segments were directly sequenced in forward and reverse directions using the BigDye 3.1 Kit and analyzed on an Applied BioSystems Genetic Analyzer 3500 XL.

Mitochondrial DNA Sequence Analysis

Sequences from forward and reverse orientations were aligned and edited with SEQUENCHER® v4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequences were aligned separately with the CLUSTAL X v1.8 multiple alignment program in BioEdit (Hall 1999). Genetic p-distance was calculated in MEGA 11 (Tamura et al. 2021). A median-joining haplotype network (Bandelt et al. 1999) was drawn using PopART (Leigh and Bryant 2015) to visualize the relationship among the haplotypes. For phylogenetic tree reconstruction, the Bayesian Information Criterion (BIC), was applied to choose the evolutionary best fit DNA substitution model settings using MEGA11 Using the (TN93 + G + I) and (K2 + G) for CR and cyt *b*, respectively (Tamura et al. 2021). BEAST X v10.5 (Drummond et al. 2012) was used to build a Bayesian tree based on the Monte Carlo Markov Chain (MCMC). We performed Bayesian inference analysis for 10 million generations, used a strict

clock model, and selected one tree every 10,000 generations with a burn-in for 5000 generations. The evolutionary tree was annotated using Tree-Annotator v1.8.1 (Bouckaert et al. 2014). The phylogenetic trees that resulted were displayed using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/ figtree/).

A 10% burn-in was used, and the convergence of all parameters was assessed using the TRACER software (part of the BEAST package) (Drummond et al. 2012). The model was summarized using a Tree Annotator with a posterior probability limit of 0.5, the height of each node in the tree set to the median height across the whole sample of trees for that type, and trees shown with FigTree, and a Bayesian Skyline Plot (BSP) was created using the Monte Carlo Markov Chain (MCMC) method in BEAST X v10.5. (Drummond et al. 2012). We calculated genetic diversity indices such as haplotype diversity (HD) and nucleotide diversity (π) using DnaSP v5.10 (Librado and Rozas 2009), estimated Tajima's D (Tajima 1989) and Fu's test (Fu 1997) and assessed the mismatch distribution.

To generate divergence time trees, the sequence of Gemsbok (*Oryx gazelle*) was chosen as an outgroup (Hassanin et al. 2012) and divergence times among blue sheep genetic lineages were estimated using the strict Clock model in BEAST v10.5 (Drummond et al. 2012) following the methodology outlined by Bouckaert et al. (2019). The Yule speciation model was employed as the tree prior. Markov Chain Monte Carlo (MCMC) runs were conducted for 10 million generations, logging parameters every 1000 generations. The initial 25% of generations were discarded as burn-in using Tracer v1.7. The resulting phylogenetic tree was visualized with FigTree v1.4.4 (Rambaut et al. 2018).

Predictor Selection for Suitability Modeling Using MaxEnt

A data set with 14 predictor variables was initially used to model the potential distribution of blue sheep. We used six bioclimatic layers obtained from the Worldclim database (Fick and Hijmans 2017); four topographic variables (elevation, slope, aspect, and terrain ruggedness index) from (SRTM) http://srtm.csi.cgiar.org/srtmdata and two LULC (Barren land and Grassland) classes from https://lpdaac.usgs.gov/products/mcd12q1v006/. We generated separate Euclidean layers for roads and rivers to test if these variables influence the distribution of blue sheep. Out of 14 variables, we further examined the collinearity of the variables (Spearman correlation coefficient $r > 0.7$), which resulted in seven uncorrelated variables for the western Himalayas and six for the eastern Himalayas ($r < 0.7$). Only a few variables were deemed biologically significant in the context of the ecological needs of blue sheep from both areas. They were used for further investigation after scaling these selected variables to 30 arc seconds, or up to 1 km near the equator. Analysis was performed in ArcGIS v10.5 and the R package 'coordinatecleaner' (Zizka et al. 2019). Model procedures were carried out with MaxEnt v3.4.4 (Phillips & Dudík 2024) after processing the blue sheep distribution and environmental variable data. The model's maximum number of iterations was set to 500, the number of cycles was set to 10, the classification of repetition was chosen as cross-validation, and the significant contribution of

selected environmental factors to the prediction of the appropriate distribution area of blue sheep was estimated.

Results

mtDNA Control Region Analysis

Genetic Diversity and Genetic Differentiation

We obtained up to 900 bp long CR sequences from 40 blue sheep samples and deposited them in GenBank (accession numbers: PP187055—PP187093 and PQ583502). We observed 193 variable sites among the samples. $T = 26.2\%$, $C = 25.7\%$, $A = 36.4\%$, and $G = 11.7\%$ constituted the standard nucleotide composition, indicating an A + T-rich area within the mitochondrial CR. Based on the CR data, 23 haplotypes were identified. We found that blue sheep had a moderate nucleotide diversity (0.0609) and a high haplotype diversity (0.97). High haplotype diversity was reported in western Himalayas- WH (0.96) and eastern Himalayas- EH (0.91). Nucleotide diversity was estimated to be 0.01833 in the WH and 0.0112 in the EH (Table 1). Using the CR, we calculated the pairwise genetic distance between the population (Table 2). A genetic distance of 22% was

Table 1 Gene diversity of control region (h), nucleotide diversity (π), Tajima's (D), and Fu's (Fs) for control region

Location	Number of samples	No. of haplotype (H)	Haplotype diversity (Hd)	Nucleotide diversity(π)	Tajima's D	Fu's test	*P*-value (Tajima's D and Fu's test)
Western Himalayas	35	19	0.96	0.0183	0.3944	0.033	$P > 0.10$
Ladakh	8	4	0.8	0.0069	1.4025	3.011	$P > 0.10$
Himachal Pradesh	6	2	0.53	0.0032	1.2188	3.696	$P > 0.10$
Uttarakhand	21	13	0.95	0.0265	1.0116	0.825	$P > 0.10$
Eastern Himalayas	5	4	0.91	0.0112	− 0.6464	1.467	$P > 0.10$
Total	40	23	0.97	0.0609	− 0.1243	5.3	$P > 0.10$

Table 2 Genetic distance of control region among blue sheep (*Pseudois nayaur*) are presented (below the diagonal), and their standard errors (above the diagonal)

	Western Himalayas	Eastern Himalayas	China Mountains
Western Himalayas	–	0.0219	0.0328
Eastern Himalayas	0.1906	–	0.0105
China Mountains	0.2223	0.0546	–

observed between the WH and the Chinese Mountains (Qilian, Helan, and Hengduan Mountains), followed by a 19% genetic distance between the EH and the WH. The lowest genetic distance of 5% was found between the EH and the Chinese Mountains.

Phylogenetic Analysis and mtDNA Network

To assess the overall mtDNA structure and to conduct the phylogenetic and network analysis, we used 40 blue sheep's sequences generated in the laboratory (accession number PP187055 to PP187093 and PQ583502) and seven additional sequences retrieved from NCBI GenBank (Supplementary Table: ST1). To have a better understanding of the evolutionary relationships, a single sequence of *Hemitragus jemlahicus* (NC020628) was selected as an out-group. Finally, a total of 47 control region sequence and one sequences of out-group were used for phylogenetic analysis. As illustrated in the CR phylogenetic tree (Fig. 2), all blue sheep populations analyzed throughout were found to be organized in one paraphyletic clade with posterior probability values (PP = 1), i.e., WH, EH, and CM (Fig. 2). The blue sheep from the western Himalayas also formed a unique clade showed in green color, distinct

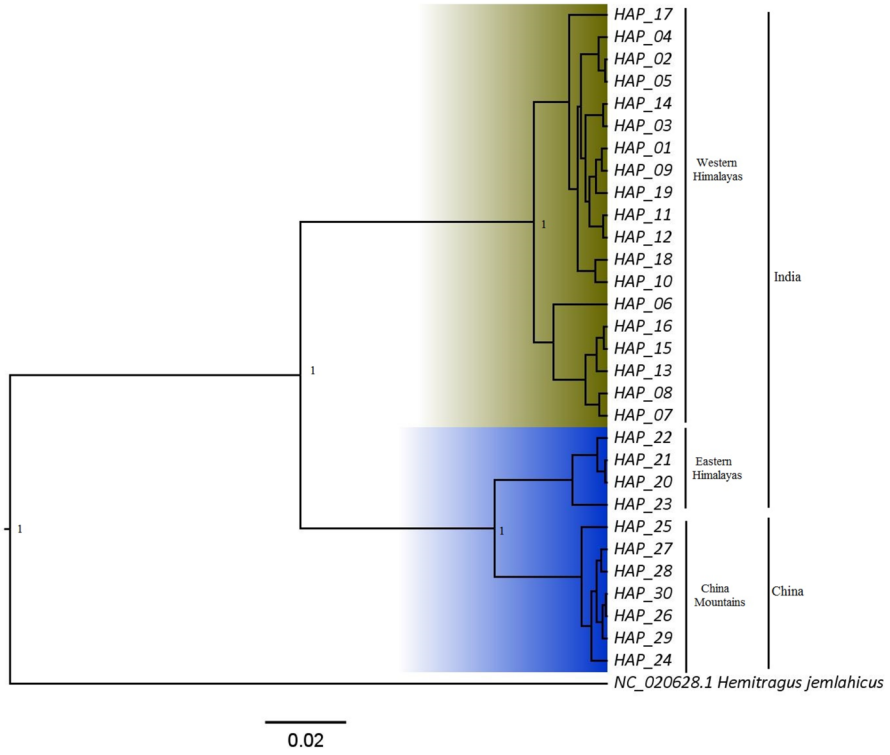

Fig. 2 Phylogenetic relationship of blue sheep species based on control region with Bayesian posterior probability values at each node

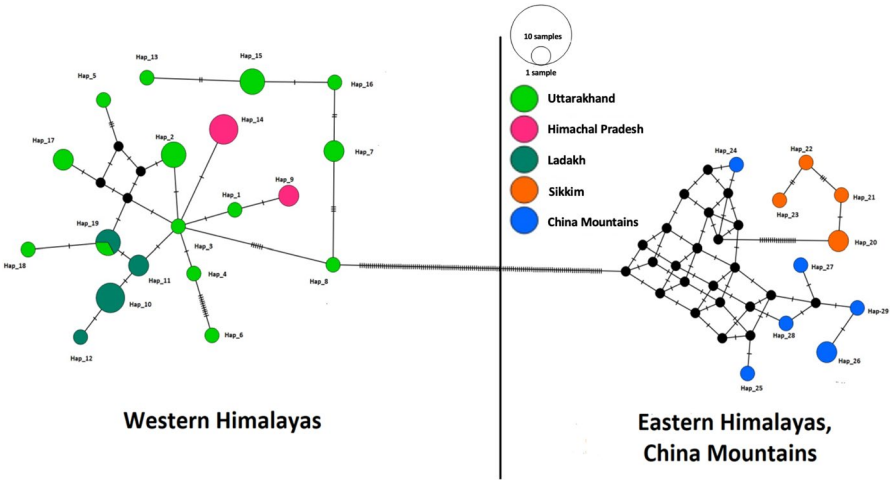

Fig. 3 Control region of blue sheep sequence-based haplotype network, where different colors represent different populations. A sequence of lines shows the number of mutational stages

from those in the CM and eastern Himalayas showed in blue color (Fig. 2). This clade from WH was found to have 19 haplotypes of blue sheep and 4 haplotypes of blue sheep from EH. The sequences from EH, which were associated with CM displayed high posterior probability value (PP = 1). In comparison, the sequences of the WH showed two groups with considerably posterior probability value (PP = 1). EH was the grouping of all the haplotypes found in the blue sheep population of China. The phylogenetic tree topology recovered from the WH and EH, CM exhibited high posterior probability value (PP = 1); based on the phylogenetic analysis, the Himalayan blue sheep formed separate clades from the Chinese blue sheep (Fig. 2).

A total of 47 control region sequences were used for network design. The distribution pattern of haplotypes across blue sheep populations was illustrated using a median-joining network, which included 30 distinct haplotypes (Fig. 3). The majority of haplotypes comprised distinct individuals. A subset of blue sheep from Uttarakhand was found to share haplotype with individuals from the Union Territory of Ladakh population, indicating a genetic connection between these populations. The blue sheep sequences from the WH formed a separate cluster and shared no haplotypes with the EH.

Demographic Analysis

The Tajima's D and Fu's tests were used to determine the demographic history of the blue sheep (Table 3). Neutrality test results under a multimodal evolution suggest a high prevalence of rare nucleotide site variants. Tajima's was negative but not statistically significant for any of the blue sheep groups, and a similar pattern was observed with Fu's. The pairwise distribution frequency within sequences was determined using a historical demographic expansion model. Multimodal plots of the mismatch distribution were found in the studied blue sheep populations found in

Table 3 Gene diversity of cyt *b* (h), nucleotide diversity (π), Tajima's (D), and Fu's (Fs)

Location	No. Of Haplotype(H)	Haplotype diversity (Hd)	Nucleotide diversity(π)	Tajima's D	Fu's test	*P*-value (Tajima's D and Fu's test)
Western Himalayas	6	0.7	0.0053	0.46066	− 5.417	$P > 0.10$
Eastern Himalayas	4	0.99	0.0261	1.36522	0.461	$P > 0.10$
Indian Himalayas Region	10	0.76	0.00994	− 0.87518	− 0.63	$P > 0.10$

IHR (Fig. 4), indicating a demographically stable population. The BSP was used to understand the historical demographic history of blue sheep that supported Tajima's D and Fu's tests for the recent population decline (Fig. 4). Moreover, BSP found that the population size did not experience any significant changes in demography for a long period (Fig. 4).

We generated 250–300 bp partial sequences of the cyt *b* gene from 37 samples of the WH region, covering three locations of Ladakh, Himachal Pradesh, and Uttarakhand (PP348784-PP348820). In addition, we included 21 sequences from China and one outgroup sequence. Thus, 59 sequences were analyzed, resulting in 38 haplotypes (Supplementary Table: ST2). The number of segregating sites in WH was 5, and the haplotype diversity (HD) was 0.70, and nucleotide diversity (π) was 0.0053. In EH, the number of segregating sites was 11, with HD = 0.99 and nucleotide diversity (π) of 0.02613. Pairwise genetic distances between populations were 4.0% between blue sheep from the WH and the CM, and 3.9% between populations from WH and EH (Table 4). However, a lower genetic

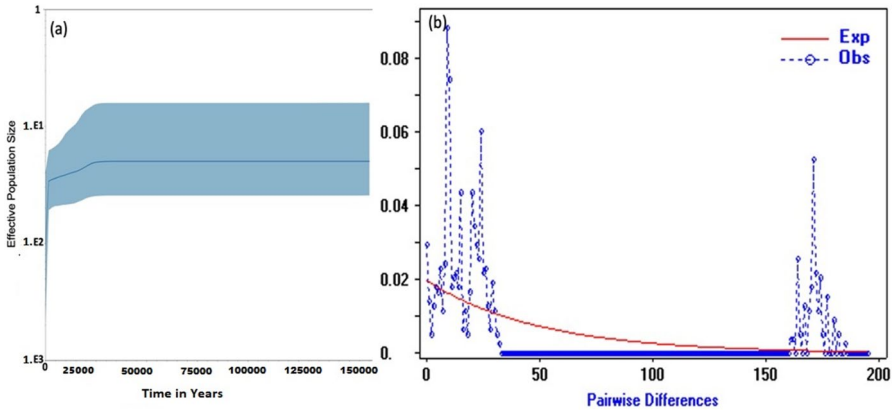

Fig. 4 Based on control region **(a)** blue Sheep's coalescent Bayesian Skyline analysis output. The dark black line indicates the median estimated effective population size, whereas the dark blue areas indicate the upper and lower bounds of the 95% higher posterior density interval of the overall population in the Indian Himalayan Region (IHR). The x-axis is time, and the y-axis is a log scale of (EPS). **b** Mismatch distributions of the blue sheep (*Pseudois nayaur*) mtDNA control region in the IHR

Table 4 Genetic distance of cyt *b* among blue sheep (*Pseudois nayaur*) are presented (below the diagonal), and their standard errors (above the diagonal)

Location	(Western Himalayas)	(Eastern Himalayas)	China Mountains
(Western Himalayas)	–	0.0115	0.011
(Eastern Himalayas)	0.0395	–	0.0086
China Mountains	0.0408	0.0288	–

distance (2.8%) was detected between EH and CM populations because of migration. A high value indicates strong genetic differentiation in blue sheep's WH and EH populations, likely due to limited gene flow. While landscape barriers are the most probable cause, other factors may possibly contribute to the restricted gene flow between these populations. The cyt *b* phylogenetic tree demonstrates that the examined blue sheep populations were grouped in paraphyletic clade with PP = 1, specifically Pamir Plateau, Tibetan Plateau, and CM (Fig. 5). The blue sheep from the Pamir Plateau and WH also formed a unique clade (showed in green color) were distinct from those in the Tibetan Plateau, CM, and the EH (showed in blue

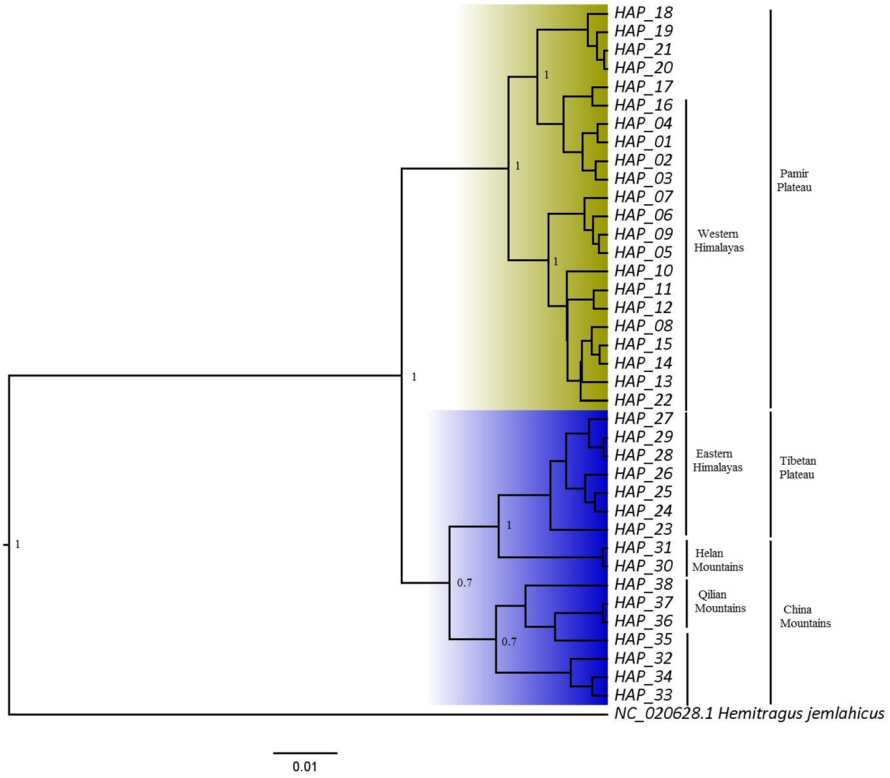

Fig. 5 Phylogenetic relationship of blue sheep (*Pseudois nayaur*) using cyt *b* with Bayesian posterior probability values at each node

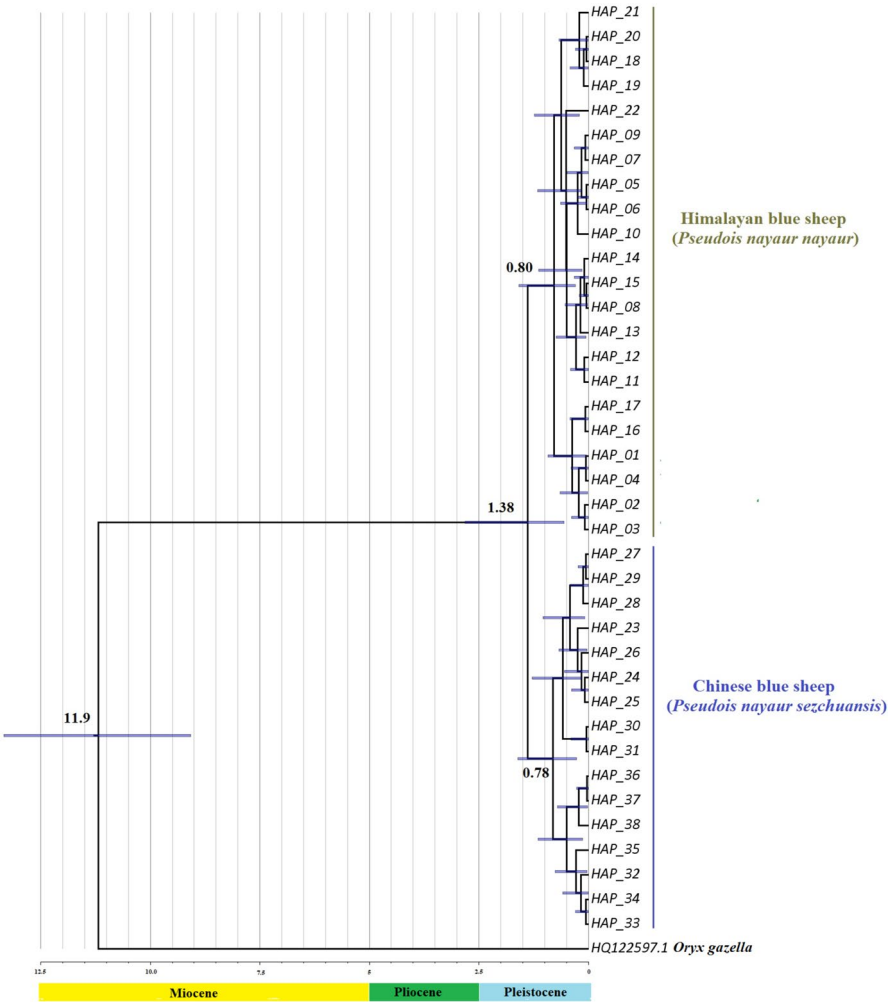

Fig. 6 Divergence dating based on a maximum credibility tree using the cyt *b* gene. The x-axis represents time in million years

color) (Fig. 5). Our divergence data indicated that the blue sheep's group diversification started during the Pleistocene, around 1.38 Mya (HPD95%: 2.84–0.48) (Fig. 6).

Response Curve Analysis of Environmental Variables

The response curves for key important factors that influence the distribution of blue sheep were presented separately for the WH and Sikkim state in the EH region (Fig. 7). The minimum temperature of the coldest month (40.4%), elevation (19.9%), and maximum temperature of the warmest month (16.5%) contributed significantly

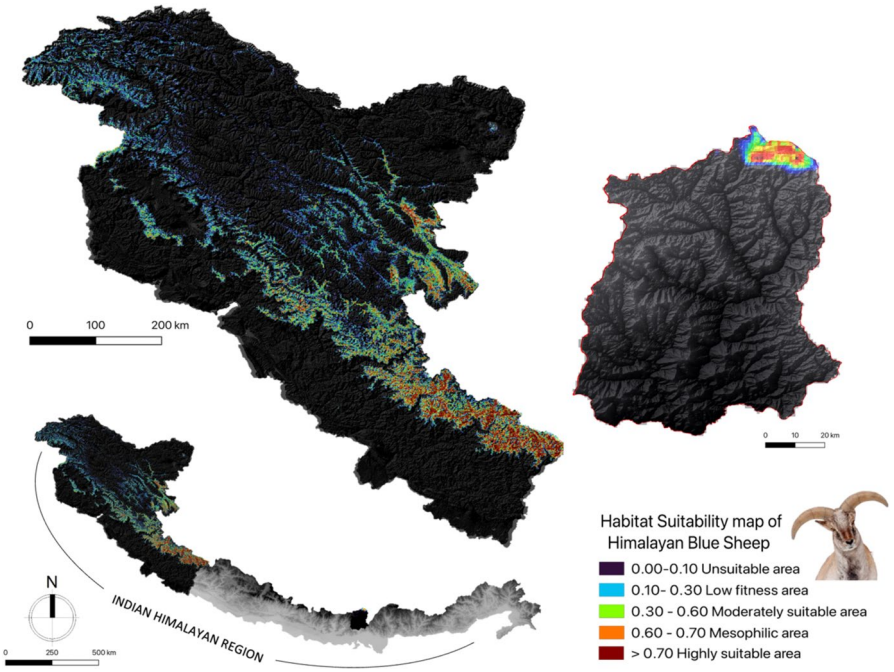

Fig. 7 Map of the suitable distribution area of blue sheep in Western Himalayas and Sikkim (Eastern Himalayas) (MaxEnt v3.4.4) (This map was created in-house using a template map of Survey of India)

to habitat selection of *P. nayaur* in WH. This species is confined to exclusive, permanent, and safe habitats at high elevations in mountains with sparse vegetation. In contrast, both factors, i.e., minimum temperature of the coldest month (35.2%) and maximum temperature of the warmest month (25.5%), contributed substantially to distribution in the EH region, followed by precipitation seasonality (13.6%) too, and had significant contributions to habitat selection of *P. nayaur* in Sikkim.

Discussion

This study was focused on the assessment of accurate spatial genetic diversity and probabilistic distribution of *P. nayaur* in the entire IHR, with coverage of western to eastern parts of the Himalaya, including Uttarakhand, Himachal Pradesh, Union Territory of Ladakh and Sikkim. Generally, blue sheep are associated with alpine and steppe mountain pastures, typically devoid of forested habitats. These open high-altitude environments provide optimal grazing conditions, but they are increasingly threatened by habitat loss, fragmentation, poaching, and other anthropogenic pressures. As a result, blue sheep populations often exhibit low genetic variation, which can hinder their ability to adapt to environmental changes and increase their vulnerability to extinction (Li and Fang 1999). The genetic variability in fragmented habitats was highlighted in this study, which has implications for regulating and

structuring conservation policies. Previous studies have proposed different genetic lineages for blue sheep in the region, with the Chinese blue sheep (*P. n. szechuanensis*) being linked to the Qinling and Hengduan Mountains. The Himalayan blue sheep (*P. n. nayaur*) being found on the Tibetan Plateau (eastern Himalayas), and the ones from Pamir Plateau (western Himalayas) having been recognized as a separate lineage (Tan et al. 2012, 2017; Gao et al. 2020). In contrast to earlier assertions based on inadequate genetic data, our genetic data exhibited that the Pamir Plateau hosts population of the Himalayan blue sheep. Furthermore, the genetic link between blue sheep populations on the Tibetan Plateau and those on the Hengduan and Qinling Mountains supports the designation of these groups as Chinese blue sheep. The robustness of the Pamir Plateau dataset identifies the importance of extensive genomic analysis in resolving taxonomic difficulties and improving the understanding of species distributions and connections within the blue sheep complex.

The blue sheep populations in the eastern and western Himalayas showed no mtDNA haplotype sharing. CR region and cyt *b* showed the paraphyletic clade in the western Himalayas. It was likely due to the WH's blue sheep habitat being dominated by high altitudes and steep slopes owing to topography, terrain, and differential climatic oscillation in contrast to EH (Fang et al. 1997). Insight of the CR region revealed high haplotype diversity (0.97) and nucleotide diversity (0.0609) in blue sheep, which indicated that several mtDNA lineages have emerged in the area by differing in their nucleotides. Moreover, the high level of CR diversity has unique traits, and the outcome of recent events in the species' evolutionary history may be due to different climatic oscillations during the late Pleistocene and Holocene periods (Joshi et al. 2022).

The high genetic distance indicated a lack of robust connectivity between WH and EH populations, highlighting a significant genetic difference (Table 2). It supports the difference in the bioclimatic condition and the topography have altered the ecological food niche that resulted in the WH and EH clades (Ranjitsinh 2017; Joshi et al. 2022). The cyt *b* region indicated that EH populations shared haplotypes with the Tibetan Plateau, hence establishing their close affinity and classification as Chinese blue sheep (*P. n. szechuanensis*), which showed a different lineage from the Hengduan mountains dwarf blue sheep (*P. schaeferi*) of China (Tan et al. 2017; Joshi et al. 2022). The phylogenetic relationship and network analysis demonstrated distinct mtDNA lineages in blue sheep from WH, as depicted in (Fig. 4.). Notably, despite potential habitat fragmentation, no haplotype sharing was observed across CM blue sheep populations, EH, and WH populations at CR level. Conversely, the EH clade exhibited similarities with the mountains of China, suggesting a potential contemporary migration history from Northeast Asia (Rawat and Tambe 2011; Bashir et al. 2018).

Tajima value was negative for the CR based neutrality test, but Fu's values were positive, i.e., statistically insignificant, which suggested the recent bottleneck effect. This result was further supported by BSP analysis, which suggested that the population had been declining during the previous few decades (Table 1). Moreover, cyt *b* analysis indicated a stable population of blue sheep supporting the findings of Dolker et al. (2023).

We suggest two distinct lineages, in Pamir Plateau-WH Himalayan blue sheep (*P. n. nayaur*) and the Tibetan Plateau—EH Chinese blue sheep (*P. n. szechuanensis)*, which was more closely associated with Helan mountain, Qilian mountains, and Hengduan (Tan et al. 2012a; Gao et al. 2020; Joshi et al. 2022; Dolker et al. 2023). The robustness of the Pamir Plateau dataset identifies the importance of extensive genomic analysis in resolving taxonomic difficulties and improving our understanding of species distributions and connections within the blue sheep complex.

Blue sheep, originating from the Tibetan Plateau and dispersed northeastwardly (Schaller 1998). Our phylogenetic divergent tree findings suggested that the Himalayan blue sheep (*P. n. nayaur*) and the Chinese blue sheep (*P. n. szechuanensis*) diverged in the Pleistocene when the Himalayan frontal range experienced a rapid uplift (Sakai et al. 2006) approximately 1.38 Mya with a 95% HPD interval of 2.84 to 0.48 Mya. This event created an insuperable barrier for the blue sheep, leading to the speculation that this species migrated to the surrounding regions and evolved into distinct clades. EH has a higher precipitation rate than WH (Polanski et al. 2014), making it moister, and this helps to sustain a continuous supply of high-quality food sources (multiple plant/grass species composition) (Yadav 2011). The distinct response curves recorded for blue sheep in the WH and EH areas demonstrate the species' exceptional adaptability to a variety of environmental conditions. Due to their direct effects on the survival, behavior, and availability of resources for *P. nayaur*, the minimum temperature of the coldest month, the maximum temperature of the warmest month, and elevation are probably significant when it comes to habitat selection in the WH. Another interpretation can be that in the WH, where elevation varies constantly, blue sheep are likely to benefit from highly specialized adaptations for rugged terrain, whereas in the EH, where precipitation has a stronger influence, access to water sources and seasonal variability likely shape habitat use patterns.

Conclusion and Implications for Conservation

The genetic data assisted in resolving taxonomy of *P. nayaur* throughout the Indian Himalayan region. The WH haplotypes were shared with the population of the Union territory of Ladakh, Himachal Pradesh, and Uttarakhand that suggested a previous maternal connectivity between these localities. The genetic variation between the populations of WH and EH blue sheep supported that WH blue sheep was Himalayan blue sheep (*P. n. nayaur*), EH and Tibet plateau blue sheep were Chinese blue sheep (*P. n. szechuanensis*). Given the differences in habitat, orography, and climate between two zones, each population should be maintained as a distinct "Evolutionary Significant Unit" within IHR and maintained as management units (MUs) for conservation management. Besides the clarification of genetic and ecological differences between the zones, our data provided better insights on evolutionary relationships and enables targeted conservation strategies that can preserve distinct evolutionary lineages and adapt to specific habitat needs, thus allowing for improved conservation planning. The newly designed control region sequence will also help

with the lineage identification of biological samples that have been seized in order to follow the cases of wildlife crimes.

Our data suggests seasonal precipitation patterns in EH affect the habitat selection of *P. nayaur* by possibly providing moister habitats and a broader array of vegetation, which improves forage quality in comparison to the drier WH region. Collectively, the blue sheep of WH and EH are able to thrive in their respective bioclimatic zones by selecting ecological food niches that would increase their fitness for their natural environment. Understanding these regional differences in habitat selection is important for successful conservation management and species conservation. Conservation efforts should take into account these ecological aspects, prioritizing habitat conservation designed for the specific needs of blue sheep populations throughout their range. This would further ensure their long-term viability in the face of potential land use change and anthropogenic pressures. We suggest initiating a collaborative spatial genetic study, employing standardized markers to identify finely detailed connecting corridors between the Pamir-WH and Tibetan plateau-EH populations. As blue sheep faces various threats, such as habitat loss, fragmentation, poaching, and competition with livestock, conducting more comprehensive and collaborative studies on the genetic and ecological aspects of blue sheep across their range and implementing effective conservation strategies based on their genetic diversity and structure may be explored.

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Author Contributions Conceptualization: SKG Sampling: DS, GS, SL, SSK Data curation: DS Formal analysis: DS, GS, TP Resources: SKG, KV Supervision: SKG, KV, SSK Writing original draft: DS, GS Writing – review & editing: SKG, KV, SSK, SL.

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Data Availability DNA sequence are available from NCBI, GenBank.

Declarations

Conflict of interest The authors declare that they have no financial support received for this study. The authors declare that they have no competing interests in this study.

Ethical approval Before conducting the field survey, the necessary approval was obtained from the concerned authority, namely, the Forest Departments of Uttarakhand, Himachal Pradesh, Ladakh, and Sikkim. We were authorized to conduct research and collect non-invasive biological samples. We used our own images in this study. The sampling location and distribution maps were created in-house using the template of the Survey of India map.

Research Involving Human and Animal Participants We did not use any live animals in this study and utilized only non-invasive samples. Hence, no Institutional Animal Ethics Committee approval was required.

References:

Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Bashir T, Bhattacharya T, Poudyal K, Sathyakumar S (2018) Preliminary observations on the cryptic fauna of Sikkim Trans-Himalaya, India. Int J Conserv Sci 9:179–184

Benton TG, Bowler DE (2012) Linking dispersal to spatial dynamics. Dispersal Ecology and Evolution. Oxford University Press, UK, pp 251–265

Bhatnagar YV, Seth CM, Takpa J et al (2019) A Strategy for conservation of the Tibetan Gazelle *Procapra picticaudata* in Ladakh. Ashoka Trust for Researc, Bangalore, pp 262–276

Bhattacharya A, Chatterjee N, Rawat GS, Habib B (2020) Blue sheep resource selection in alpine grasslands of a western himalayan landscape – A point process approach. Zool Stud 59:1–15. https://doi.org/10.6620/ZS.2020.59-11

Bouckaert R, Heled J, Kühnert D et al (2014) BEAST 2: A software platform for bayesian evolutionary analysis. PLoS Comput Biol 10:e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bouckaert R, Vaughan TG, Barido-Sottani J, et al (2019) BEAST 2.5: an advanced software platform for Bayesian evolutionary analysis. PLoS Comput Biol 15:e1006650. https://doi.org/10.1371/journal.pcbi.1006650

Dalui S, Khatri H, Singh SK et al (2020) Fine-scale landscape genetics unveiling contemporary asymmetric movement of red panda (Ailurus fulgens) in Kangchenjunga landscape, India. Sci Rep 10:1–13. https://doi.org/10.1038/s41598-020-72427-3

Dolker S, Jabin G, Singh SK et al (2023) Landscape genetics identified conservation priority areas for blue sheep (Pseudois nayaur) in the Indian Trans-Himalayan region. Sci Rep 13:18152. https://doi.org/10.1038/s41598-023-44823-y

Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol 29:1969–1973. https://doi.org/10.1093/molbev/mss075

Joshi BD, Jabin G, Sharief A et al (2020) Genetic evidence for allopatric speciation of the *Siberian ibex Capra sibirica* in India. Endanger Species Res 42:1–5. https://doi.org/10.3354/esr01034

Fang X-M, Li J-J, Van der Voo R et al (1997) A record of the blake event during the last interglacial paleosol in the western Loess Plateau of China. Earth Planet Sci Lett 146:73–82. https://doi.org/10.1016/S0012-821X(96)00222-1

Farooquee NA, Nautiyal A (1999) Traditional knowledge and practices of Bhotiya pastoralists of Kumaon Himalaya: the need for value addition. Int J Sustain Dev World Ecol 6:60–67. https://doi.org/10.1080/13504509.1999.9728472

Fick SE, Hijmans RJ (2017) WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. Int J Climatol 37:4302–4315. https://doi.org/10.1002/joc.5086

Fu Y-X (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925. https://doi.org/10.1093/genetics/147.2.915

Gao H, Nannan LI, Huang Y et al (2020) Taxonomic status of Chinese blue sheep (*Pseudois nayaur*): new evidence of a distinct subspecies. Integr Zool 15:202–212. https://doi.org/10.1111/1749-4877.12422

Ghosh A, Thakur M, Singh SK et al (2022) The Sela macaque (Macaca selai) is a distinct phylogenetic species that evolved from the Arunachal macaque following allopatric speciation. Mol Phylogenet Evol 174:107513. https://doi.org/10.1016/j.ympev.2022.107513

Gupta SK, Kumar A, Hussain SA (2013) Extraction of PCR-amplifiable DNA from a variety of biological samples with uniform success rate. Conserv Genet Resour 5:215–217. https://doi.org/10.1007/s12686-012-9772-9

Hall TA (1999) BioEdit: a user friendly biological seque. Nucleid Acids Symp. Ser.pp 95–98

Harris RB (2014) Wildlife Conservation in China Preserving the Habitat of China's Wild West. Taylor and Francis, New York

Hassanin A, Delsuc F, Ropiquet A et al (2012) Pattern and timing of diversification of Cetartiodactyla (Mammalia, *Laurasiatheria*), as revealed by a comprehensive analysis of mitochondrial genomes. Comptes Rendus - Biol 335:32–50. https://doi.org/10.1016/j.crvi.2011.11.002

Joshi BD, Kumar V, De R et al (2022) Mitochondrial cytochrome b indicates the presence of two paraphyletic diverged lineages of the blue sheep *Pseudois nayaur* across the Indian Himalaya: conservation implications. Mol Biol Rep 49:11177–11186. https://doi.org/10.1007/s11033-022-07832-0

Suryawanshi KR (2008) Overwintering strategies and demographic response of bharal (*Pseudois nayaur*) to livestock grazing and removal, in Kibber wildlife sanctuary. National centre for biological sciences

Leigh JW, Bryant D (2015) <scp>popart</scp> : full-feature software for haplotype network construction. Methods Ecol Evol 6:1110–1116. https://doi.org/10.1111/2041-210X.12410

Li J, Fang X (1999) Uplift of the Tibetan plateau and environmental changes. Chinese Sci Bull 44:2117–2124. https://doi.org/10.1007/BF03182692

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452. https://doi.org/10.1093/bioinformatics/btp187

Manel S, Schwartz MK, Luikart G, Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. Trends Ecol Evol 18:189–197. https://doi.org/10.1016/S0169-5347(03)00008-9

Mishra C, Van Wieren SE, Ketner P et al (2004) Competition between domestic livestock and wild bharal *Pseudois nayaur* in the Indian Trans-Himalaya. J Appl Ecol 41:344–354. https://doi.org/10.1111/j.0021-8901.2004.00885.x

Moritz C (1994) Defining 'evolutionarily significant units' for conservation. Trends Ecol Evol 9:373–375. https://doi.org/10.1016/0169-5347(94)90057-4

Namgail T, Fox JL, Bhatnagar YV (2009) Status and distribution of the near threatened tibetan argali ovis ammon hodgsoni in Ladakh, India: effect of a hunting ban. Oryx 43:288–291. https://doi.org/10.1017/S0030605308000264

Namgail T, Fox JL, Bhatnagar YV (2007) Habitat shift and time budget of the Tibetan argali: the influence of livestock grazing. Ecol Res 22:25–31. https://doi.org/10.1007/s11284-006-0015-y

Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evol Biol Cent Uppsala Univ 2:1–2

Polanski S, Fallah B, Befort DJ et al (2014) Regional moisture change over India during the past Millennium: a comparison of multi-proxy reconstructions and climate model simulations. Glob Planet Change 122:176–185. https://doi.org/10.1016/j.gloplacha.2014.08.016

Rambaut A, Drummond AJ, Xie D et al (2018) Posterior summarization in bayesian phylogenetics using tracer 1.7. Syst Biol 67:901–904. https://doi.org/10.1093/sysbio/syy032

Ranjitsinh MK (2017) A Life with Wildlife: From Princely India to the Present. Haper collins, UK

Rawat GS, Tambe S (2011) Sikkim Himalaya: unique Features of Biogeography and Ecology. Biodivers, Sikk

Saini D, Yadav P, Kolipakam V et al (2023) The complete mitogenome of blue sheep (*Pseudois nayaur*) from the Indian Himalayan region and its comparative phylogenetic relationship with other related species. Anim Gene 29:200155. https://doi.org/10.1016/j.angen.2023.200155

Sakai H, Sakai H, Yahagi W et al (2006) Pleistocene rapid uplift of the Himalayan frontal ranges recorded in the Kathmandu and Siwalik basins. Palaeogeogr Palaeoclimatol Palaeoecol 241:16–27. https://doi.org/10.1016/j.palaeo.2006.06.017

Schaller GB (1998) Wildlife of the Tibetan Steppe. University Chicago Press, Chicago

SRTM SRTM Data. http://srtm.csi.cgiar.org/srtmdata

Steven J. Phillips, Miroslav Dudík RES (2024) Maxent software for modeling species niches and distributions (Version 3.4.1)

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595. https://doi.org/10.1093/genetics/123.3.585

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120

Tan S, Wang Z, Jiang L et al (2017) Molecular phylogeny and phylogeography of genus Pseudois (Bovidae, Cetartiodactyla): new insights into the contrasting phylogeographic structure. Ecol Evol 7:7047–7057. https://doi.org/10.1002/ece3.3269

Tan S, Zou D, Tang L et al (2012) Molecular evidence for the subspecific differentiation of blue sheep (*Pseudois nayaur*) and polyphyletic origin of dwarf blue sheep (*Pseudois schaeferi*). Genetica 140:159–167. https://doi.org/10.1007/s10709-012-9667-4

Verma SK, Singh L (2003) Novel universal primers establish identity of an enormous number of animal species for forensic application. Mol Ecol Notes 3:28–31. https://doi.org/10.1046/j.1471-8286.2003.00340.x

Wang X-M, Cao L-R, Liu Z-S, Fang S-G (2006) Mitochondrial DNA variation and matrilineal structure in blue sheep populations of Helan Mountain, China. Can J Zool 84:1431–1439. https://doi.org/10.1139/z06-137

Wang XM, Peng JT, Zhou HM (2000) Preliminary observations on the distribution and status of dwarf blue sheep *Pseudois schaeferi*. Oryx 34:21–26. https://doi.org/10.1046/j.1365-3008.2000.00089.x

Yadav SS (2011) Crop adaptation to climate change. Wiley, Hoboken

Yu W, Xiao-Ming W (2003) Population ecology of dwarf blue sheep (*Pseudois schaeferi*). Biodivers Sci 11:59–62. https://doi.org/10.17520/biods.2003008

Zeng B, Xu L, Yue B et al (2008) Molecular phylogeography and genetic differentiation of blue sheep Pseudois nayaur szechuanensis and *Pseudois schaeferi* in China. Mol Phylogenet Evol 48:387–395. https://doi.org/10.1016/j.ympev.2008.05.010

Zizka A, Silvestro D, Andermann T et al (2019) CoordinateCleaner: Standardized cleaning of occurrence records from biological collection databases. Methods Ecol Evol 10:744–751. https://doi.org/10.1111/2041-210X.13152

Authors and Affiliations

Deepesh Saini[1] · Gaurav Sonker[1] · Tushar Parab[1] · Vishnupriya Kolipakam[1] · Sambandam Sathyakumar[1] · Salvador Lyngdoh[1] · Sandeep Kumar Gupta[1]

✉ Sandeep Kumar Gupta
skg@wii.gov.in

[1] Wildlife Institute of India, Chandrabani, Dehradun, Uttarakhand 248001, India

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SHORT COMMUNICATION

Unveiling genetic variation among Bharal (*Pseudois nayaur*) in the Indian Himalayas using nuclear markers

Deepesh Saini[1] · Gaurav Sonker[1] · Pooja Pant[1] · Vishnupriya Kolipakam[1] · Sambandam Sathyakumar[1] · Salvador Lyngdoh[1] · Sandeep Kumar Gupta[1]

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Abstract
Understanding the genetic structure of wild ungulate populations is essential for informed conservation planning, particularly in ecologically sensitive and topographically complex landscapes such as the Himalayas. We investigated the genetic variation in Bharal (*Pseudois nayaur*) populations from the western (WH) and eastern Himalayas (EH) using eight microsatellite loci. Our analysis revealed significant genetic divergence between WH and EH populations, with a Nei's genetic distance of 0.91 and a pairwise FST value of 0.14, indicating their delineation as distinct lineages. WH populations showed greater genetic affinity with the Himalayan Bharal (*P. n. nayaur*). In contrast, EH populations were closely related to the Chinese Bharal (*P. n. szechuanensis*) of the Tibetan Plateau. Hence, WH and EH Bharal represent distinct Evolutionarily Significant Units (ESUs) and should be managed as separate Management Units (MUs). It further highlighted the need for region-specific conservation strategies to safeguard the genetic integrity and ecological resilience of Bharal populations across the Indian Himalayan Region.

Keywords Phylogenetics · Molecular taxonomy · Indian himalayan region (IHR) · Conservation genetics · Ungulates

Introduction

The Bharal or blue sheep (*Pseudois nayaur*) is a keystone herbivore of the high-altitude's low-productivity ecosystems of the Indian Himalayan Region (IHR) with a distribution extending across the Tibetan Plateau, encompassing India, China, Bhutan, and Nepal (Oli 1996; Mishra 1997; Chetri et al. 2017; Pal et al. 2022a, b; Joshi et al. 2022). It is currently listed as 'Least Concern' in IUCN Red List; however, its populations are exhibiting a declining trend due to increasing anthropogenic pressures and climate-induced habitat alterations. These stressors not only reduce suitable habitat but also intensify interactions with domestic livestock, facilitating disease transmission and potential genetic admixture. Furthermore, illegal hunting for bushmeat exacerbates the threats facing wild populations (Mishra et al. 2004; Suryawanshi et al. 2010; Bhattacharya et al. 2020; Dolker et al. 2023).

Bharal serve as the primary wild prey for apex predators including the 'Vulnerable' snow leopard (*Panthera uncia*) and the dhole (*Cuon alpinus chanco*), underscoring their ecological significance in sustaining predator populations (Shrestha and Wegge 2008; Aryal et al. 2014; Lyngdoh et al. 2014; Shrestha et al. 2018). As such, conserving Bharal populations is integral to maintaining the functional integrity of alpine ecosystems. The species exhibits extensive morphological and ecological adaptations to the harsh conditions of the Himalayan and trans-Himalayan landscape (Oli 1996; Namgail et al. 2004; IUCN 2017; Bhattacharya et al. 2020; Pal et al. 2022a, b; Joshi et al. 2022; Dolker et al. 2023), necessitating regionally adaptive management strategies for the conservation of isolated populations (Dalui et al. 2020).

Despite its broad range and ecological importance, the genetic structure and evolutionary history of the western (WH) and eastern (EH) Himalayas' Bharal remain poorly characterized (Saini et al. 2025). Molecular phylogenetics has played a crucial role in identifying cryptic lineages among high-altitude ungulates (Namgail et al. 2004; Hassanin et al. 2012). A recent study based on mitochondrial

✉ Sandeep Kumar Gupta
skg@wii.gov.in

[1] Wildlife Institute of India, Chandrabani, Dehradun, Uttarakhand, India

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DNA indicated substantial genetic divergence between WH Bharal and those from the Tibetan Plateau, suggesting the existence of distinct evolutionary lineages. Specifically, WH populations are more closely aligned with the Himalayan Bharal (*P. n. nayaur*), whereas EH populations show greater similarity to the Chinese Bharal (*P. n. szechuanensis*) (Saini et al. 2025).

This divergence likely reflects historical biogeographic processes, such as glacial refugia, riverine barriers, and topographic complexity (Li et al. 2005; Tan et al. 2017; Bhattacharya et al. 2020). However, the ecological and environmental factors underlying these patterns and the extent of population differentiation across the IHR remain to be fully elucidated.

Genetic characterization of Bharal populations is useful in formulating conservation strategies, particularly in landscapes with habitat fragmentation and local adaptation (Wan et al. 2018; Dalui et al. 2020; Ram et al. 2021; Dolker et al. 2023). While mitochondrial DNA provides valuable insights into maternal lineages, it offers a limited perspective on broader genetic processes such as gene flow, allelic diversity, and population structure. The microsatellite markers offer high-resolution information on individual and population-level variation, enabling the identification of Evolutionarily Significant Units (ESUs) and Management Units (MUs) (Li et al. 2005; Frankham 2012; Funk et al. 2012).

In this study, we examine the nuclear genetic diversity and population structure of Bharal across WH and EH regions using microsatellite markers. Our objective is to validate the genetic distinctiveness of these populations and to inform region-specific conservation planning in the Indian Himalayas. By integrating genetic data with ecological context, we aim to understand the evolutionary forces shaping Bharal diversity and inform strategies for their long-term conservation.

Materials and methods

Sample collection and preparation

A total of 150 fecal samples were collected from Bharal (*Pseudois nayaur*) populations across the Indian Himalayas between July 2022 and July 2023. Due to degradation and suboptimal condition, 47 samples were selected for DNA extraction and subsequent analysis. Of these, 31 samples originated from the western Himalayas including the Union Territory of Ladakh, Uttarakhand, and Himachal Pradesh and 16 samples were collected from the eastern Himalayas (Sikkim) (Fig. 1). Additionally, two tissue samples were opportunistically obtained from naturally dead individuals in Uttarakhand and preserved in 80% ethanol.

Fecal samples were collected using sterile gloves and stored in 2 mL microcentrifuge tubes containing either 80% ethanol or silica gel desiccant to preserve DNA integrity. Samples were kept at −4 °C until DNA extraction. To minimize the likelihood of sampling genetically related individuals, fecal samples were collected at intervals of at least 300–500 m, across different locations and time points.

Genomic DNA was extracted from fecal samples using the guanidine hydrochloride-silica (GuHCl-silica) method (Gupta et al., 2013), and from tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. DNA was eluted in 50 µL of elution buffer. Quality and concentration of extracted DNA were assessed using 0.8% agarose gel electrophoresis stained with ethidium bromide (EtBr) and quantified using a QIAxpert spectrophotometer (Qiagen, Hilden, Germany). All DNA extracts were diluted to a working concentration of approximately 20 ng/µL for PCR amplification. Metadata, including sample locations and tissue types, is provided in Supplementary Table ST1.

Species identification, PCR amplification

Species was confirmed by sequencing a partial fragment of the mitochondrial control region using primer pair, Forward: 5′-CTATAGCCTCACTATCAGCAC-3′ and Reverse: 5′-AGCACAGTTATGTGTGAGC-3′ (Saini et al. 2025). All genetically verified samples were subsequently genotyped using eight polymorphic microsatellite loci (Supplementary Table ST2) selected from previously published sources for their suitability in ungulate genetics (Chang et al. 2012; Gaur et al. 2003; Jones et al., 2002; Poetsch et al., 2001; Vaiman et al., 1992; Tan et al. 2012a; Zeng et al. 2008).

PCR amplifications were conducted in 20 µL reaction volumes containing 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0), 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 3 pmol of each primer, 0.6 units of DreamTaq DNA Polymerase (Thermo Fisher Scientific, USA), and 4 µL of template DNA. Thermocycling conditions included an initial denaturation at 95 °C for 5 min; followed by 38 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s, and extension at 72 °C for 90 s; with a final extension at 72 °C for 10 min. Negative and positive controls were included in all reactions.

Amplification success was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide (0.5 mg/mL), and visualized using a UV transilluminator (Analytik Jena, Germany). Successful PCR products were enzymatically purified using Exonuclease I and alkaline phosphatase at 37 °C for 20 min, followed by enzyme inactivation at 85 °C

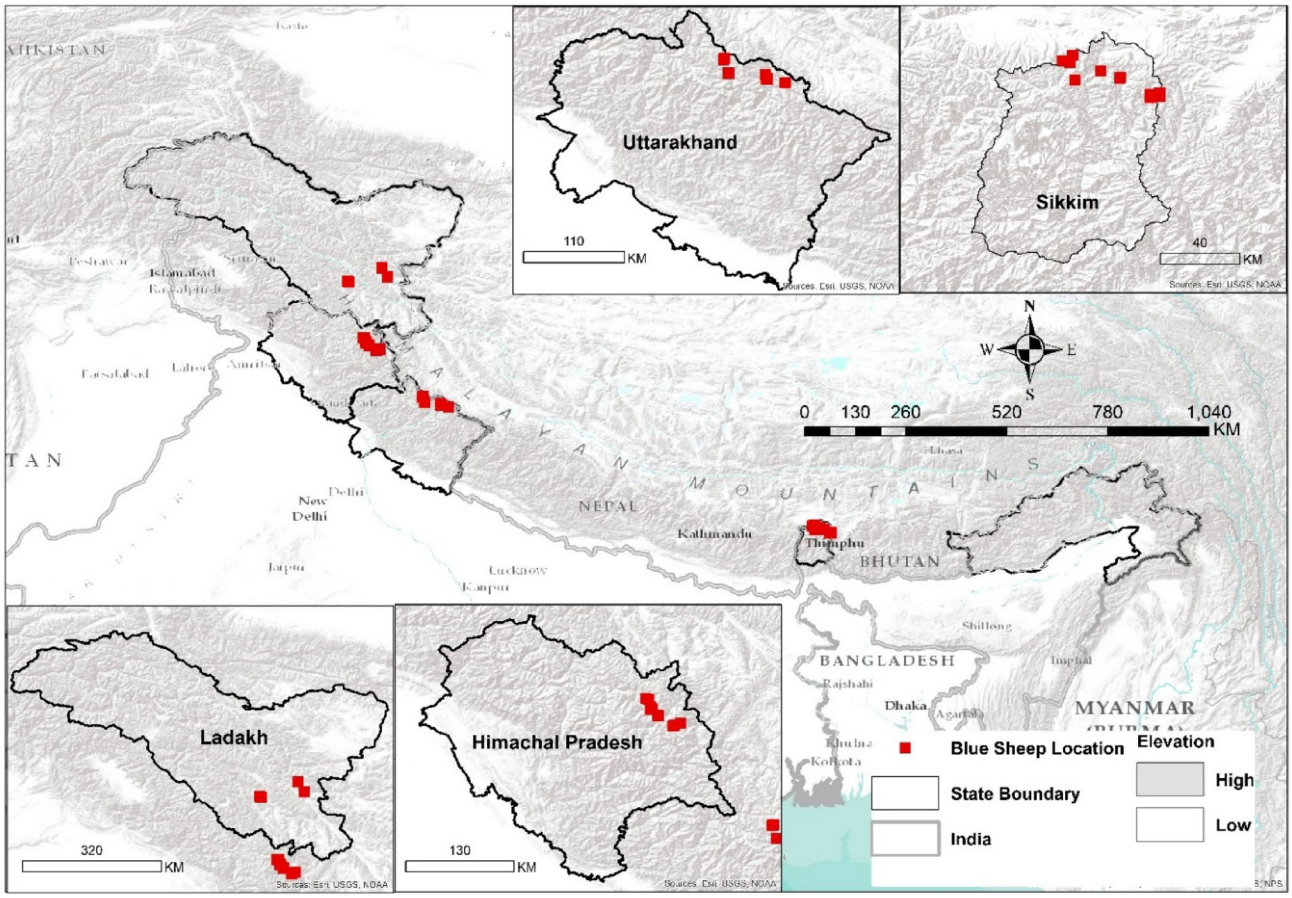

Fig. 1 Distribution and sampling locations for the current study. Yellow circles indicate the location of the sampling sites

for 15 min. Purified products were sequenced bidirectionally using the BigDye Terminator v3.1 Cycle Sequencing Kit and analyzed on a 3500 XL Genetic Analyzer (Applied Biosystems, USA).

Microsatellite analysis

Microsatellite genotyping was performed on genetically confirmed Bharal samples. Of the 18 screened loci, eight yielded consistent amplification (Supplementary Table ST3). Fluorescently labeled PCR products were genotyped on a 3500 XL Genetic Analyzer (Applied Biosystems), and allele sizes were scored using GeneMapper v4.1 (Applied Biosystems).

The selected loci were chosen based on their short amplicon sizes, high amplification success, lack of genotyping errors, and high discriminatory power, as verified using MICROCHECKER v2.2.3 (Van Oosterhout et al. 2004). These criteria were essential to minimize the risk of sampling the same individuals more than once. Unique individual identification was performed using GENECAP v1.2.2 (Wilberg and Dreher 2004). The polymorphic information content (PIC) of each locus was estimated using CERVUS v3.0 (Kalinowski et al. 2007).

Population genetic diversity indices including the number of observed alleles (Na), effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and Wright's inbreeding coefficient (FIS) were calculated using AMOVA in GenAlEx v6.5 (Peakall and Smouse 2012).

To assess population structure, we used two approaches: a Bayesian clustering algorithm implemented in STRUCTURE v2.3.4 (Pritchard 2007), and a multivariate Discriminant Analysis of Principal Components (DAPC). STRUCTURE analyses were conducted using a burn-in of 10,000 iterations followed by 100,000 MCMC repetitions, with 10 independent replicates for each K value. The optimal number of clusters (K) was determined using the ΔK method (Evanno et al. 2005). Individuals were assigned to clusters based on a minimum membership threshold of $q \geq 0.80$ (Lecis et al. 2006; Mukesh et al., 2013).

Results

Partial control region sequences were successfully obtained from 47 fecal samples (Supplementary Table ST1). These matched with *Pseudois nayaur* in the BLAST search against the NCBI GenBank. Consistent amplification (≥70% success) was obtained from these samples for eight microsatellite loci, which were used in further analysis (Table 1). These loci exhibited high informativeness, with polymorphic information content (PIC) values exceeding 0.5.

Genetic diversity indices revealed notable differentiation between western Himalayan (WH) and eastern Himalayan (EH) populations. Mean observed allele numbers (Na) ranged from 6.21 ± 0.85 in WH to 3.88 ± 0.44 in EH, while effective allele numbers (Ne) ranged from 3.98 ± 0.83 (WH) to 2.89 ± 0.41 (EH). Mean observed heterozygosity (Ho) was 0.57 ± 0.09 in WH and 0.63 ± 0.06 in EH, while mean expected heterozygosity (He) was 0.67 ± 0.05 in WH and 0.61 ± 0.04 in EH. Overall, across both populations, mean Ho and He were 0.65 ± 0.03 and 0.64 ± 0.03, respectively, with a combined Na of 5.0 ± 0.54 (Table 1). Shannon's Information Index (I) values indicated a relatively stable population structure. Fixation index (F) values ranged from −0.067 to 0.585, generally falling within moderate levels of genetic differentiation.

Bayesian clustering analysis in STRUCTURE revealed the presence of two distinct genetic clusters, as indicated by the highest ΔK value at K = 2 (Fig. 2). Individuals from the WH (n = 31) were primarily assigned to Cluster I, while those from the EH (n = 16) grouped into Cluster II. Individuals from Ladakh, Himachal Pradesh, and Uttarakhand demonstrated consistent shorting to the WH cluster. Discriminant Analysis of Principal Components (DAPC) corroborated this pattern, identifying two major genetic clusters congruent with geographic separation (Fig. 3).

A Mantel test indicated significant isolation by distance (IBD) across the sampled populations (r = 0.381; P < 0.05), supporting a geographic component to genetic divergence (Fig. 4). AMOVA results showed that 22% of the total genetic variance was attributed to differentiation between lineages corresponding to *P. n. szechuanensis* (from the Qinling and Hengduan Mountains and Tibetan Plateau) and *P. n. nayaur* (Western Himalayan region). A further 12% of variation was due to differences among individuals within populations, while 66% of the variation occurred within individuals, highlighting substantial individual-level genetic diversity (Table 2).

Discussion

Habitat loss, fragmentation, poaching, and other anthropogenic pressures have been shown to reduce genetic diversity in Bharal populations, potentially diminishing their adaptive capacity and increasing extinction risk (Li and Fang 1999; Wang et al. 2006; Wan et al. 2018; Dolker et al. 2023; Saini et al. 2025). Our study highlights the persistence of genetic variability within fragmented habitats, providing critical insight into the population structure of *Pseudois nayaur* and informing future conservation policy.

Phylogenetically, *Pseudois* shares a close evolutionary relationship with other Caprinae taxa, including *Capra*

Table 1 Genotyping parameters of used microsatellite loci

	Locus	*N*	Na	Ne	I	Ho	He	F	PIC
P. n. nayaur	FS120	30	11.000	8.612	2.254	0.633	0.884	0.283	0.881
	FS59	29	3.000	2.600	1.013	0.793	0.615	− 0.289	0.623
	INRA	31	7.000	3.070	1.429	0.419	0.674	0.378	0.803
	RT1	31	7.000	5.754	1.825	0.839	0.826	− 0.015	0.786
	SY112A	31	5.000	2.934	1.234	0.226	0.659	0.657	0.610
	SY58	30	4.000	1.671	0.754	0.167	0.402	0.585	0.579
	CA18	31	6.000	5.125	1.708	0.903	0.805	− 0.122	0.814
	T156	31	6.000	2.103	1.073	0.613	0.524	− 0.169	0.632
P. n. szechuanensis	FS120	13	6.000	5.365	1.728	0.769	0.814	0.055	0.881
	FS59	16	3.000	2.169	0.865	0.875	0.539	− 0.623	0.623
	INRA	16	5.000	3.048	1.321	0.625	0.672	0.070	0.803
	RT1	14	4.000	3.379	1.301	0.857	0.704	− 0.217	0.786
	SY112A	16	4.000	1.707	0.822	0.375	0.414	0.094	0.610
	SY58	16	2.000	1.882	0.662	0.500	0.469	− 0.067	0.579
	CA18	16	4.000	3.048	1.212	0.625	0.672	0.070	0.814
	T156	16	3.000	1.015	1.015	0.438	0.607	0.280	0.632
Mean		22.93	5.000	3.439	1.263	0.604	0.643	0.659	**0.7257**
SE		1.967	0.548	0.469	0.110	0.058	0.037	0.037	

N number of samples, *na* number of alleles, *ne* no. of effective alleles, *i* shannon's information index, *HO* observed heterozygosity; *HE* expected heterozygosity, *f* fixation index, *pic* polymorphic information content

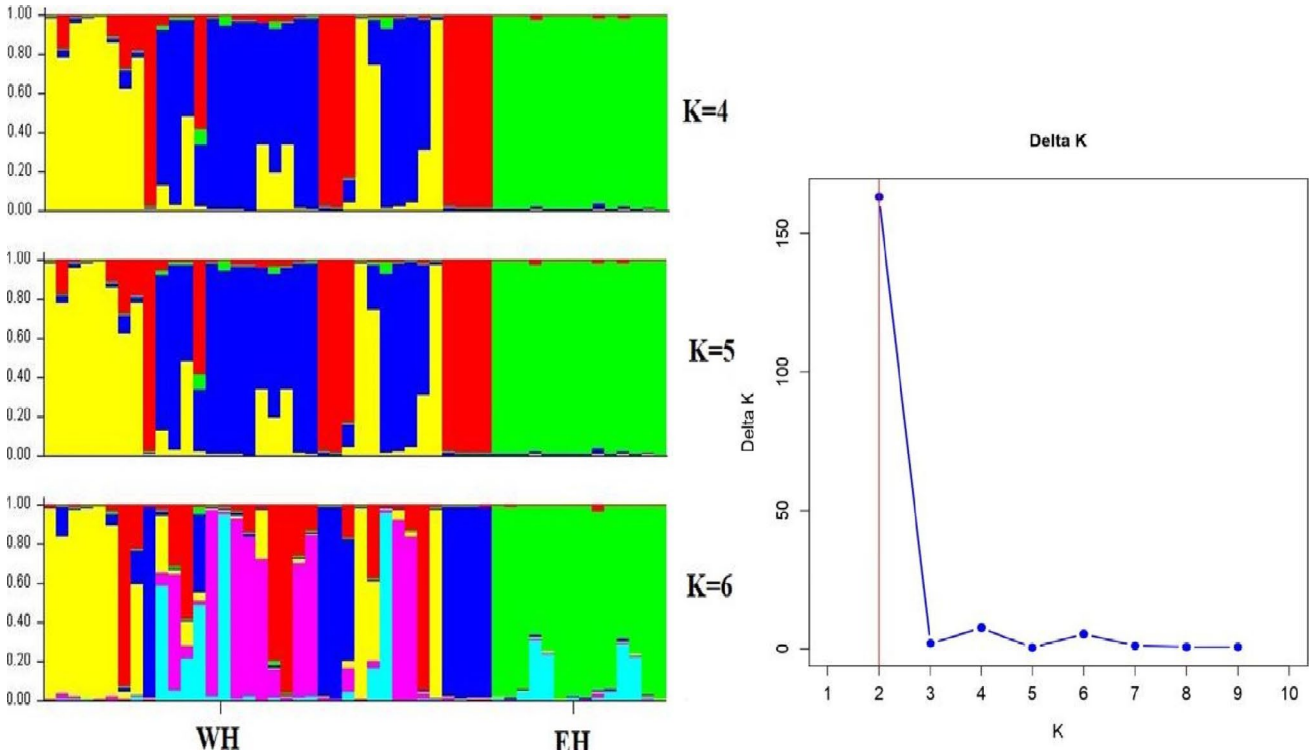

Fig. 2 Bharal population clustering patterns using Bayesian clustering at K2, K3, K4, K5, and K6; population-level assignment (cluster 1: Western Himalaya, cluster 2: Eastern Himalaya); Mean L (K), A (i) (ii). For every K value, the ad hoc quantity (delta K) is calculated across 20 runs. STRUCTURE v 2.3.4 was used to conduct the structural analysis

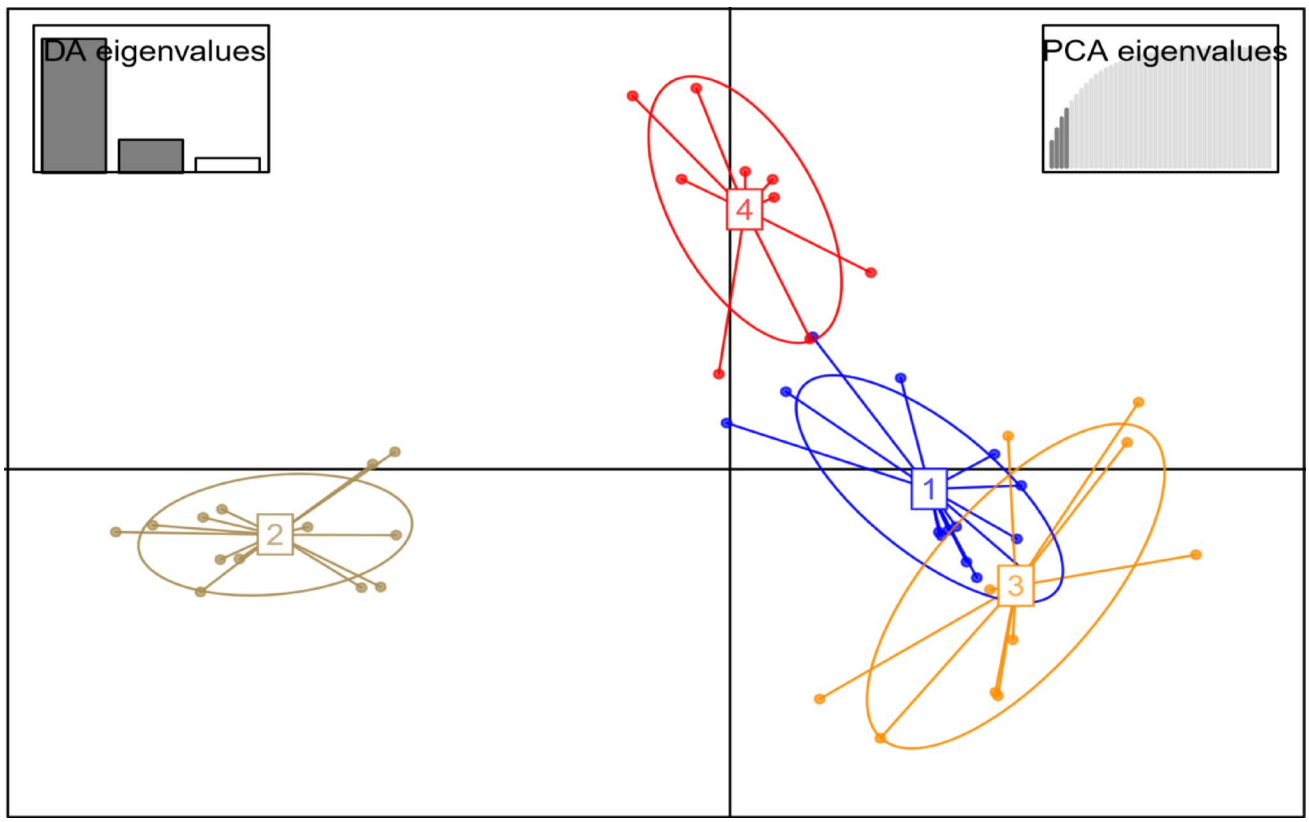

Fig. 3 Non-Bayesian pattern of bharal with DAPC

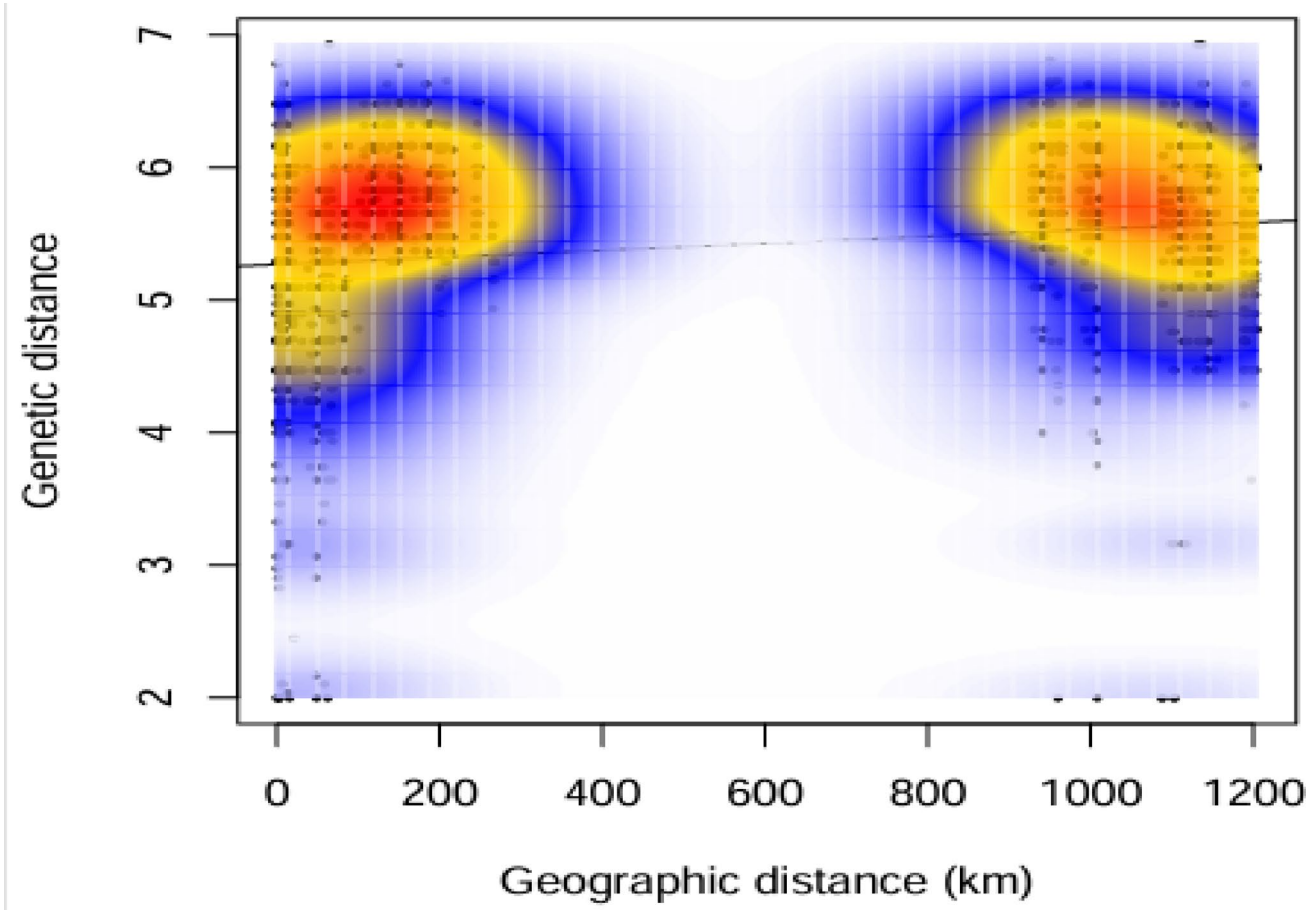

Fig. 4 The Mantel test result for IBD (Isolation by Distance) in the bharal population is displayed in a scatterplot along with the relevance of geographic distance on genetic distance ($r = 0.381$). Warmer colours indicate higher densities and demonstrate the link between the distance matrices. Colour indicates the relative density of a spot. IBD was carried out using the Adegenet version 1.3.4 program in R.4.0 software

Table 2 Analysis of molecular variance (AMOVA)

Source	df	SS	MS	Estimated Variation	%
Among Populations	1	35.615	35.615	0.770	22%
Among Individual	45	140.853	3.130	0.411	12%
Within Individual	47	108.500	2.309	2.309	66%

df degrees of freedom, *SS* sum of squares, *MS* mean square, *%* Percentage of total variance

hircus, *Capra ibex*, and various *Ovis* species (Geist 1987; Luenser et al. 2005). Caprinae as a whole form a sister clade to Boselaphini (*Boselaphus tragocamelus*) and Bovini (*Bos gaurus*, *Bubalus bubalis*), underscoring the evolutionary distinctiveness of *P. nayaur* within Bovidae (Saini et al. 2023). This distinct phylogenetic position reflects the unique evolutionary trajectory of Bharal and emphasizes its conservation value across its distributional range.

Our microsatellite-based analysis revealed two distinct genetic lineages of Bharal across the Indian Himalayan Region (IHR), corresponding to the Chinese Bharal (*P. n. szechuanensis*) found in the Qinling, Hengduan, and Tibetan Plateau, and the Himalayan Bharal (*P. n. nayaur*) of the Pamir Plateau. The WH populations were genetically aligned with *P. n. nayaur*, while EH populations showed greater affinity with the *szechuanensis* lineage. These findings are consistent with mitochondrial data from prior studies (Saini et al. 2023; Gao et al. 2020) and suggest the presence of a significant biogeographic barrier within the IHR that restricts gene flow and promotes lineage divergence.

The distinct clustering of EH and WH individuals in DAPC and STRUCTURE analyses, with no evidence of admixture, further supports this division. The genetic distance test (IBD) also indicated weak connectivity between regions, reflecting limited dispersal and substantial genetic differentiation (Fig. 4). Such divergence is likely shaped by historical and ecological drivers, including topographic barriers and bioclimatic heterogeneity, which have contributed to the formation of region-specific ecological (Ranjitsinh 2017; Joshi et al. 2022; Saini et al. 2025).

Our findings reinforce the presence of two evolutionarily significant units: the western Himalayan Bharal (*P. n. nayaur*) and the eastern Himalayan Bharal (*P. n. szechuanensis*),

with the latter showing closer genetic proximity to populations in the Helan, Qilian, and Hengduan Mountains (Tan et al. 2012b; Benton and Bowler 2012; Bhattacharya et al. 2020; Gao et al. 2020; Joshi et al. 2022; Dolker et al. 2023). The robustness of our dataset from the Pamir Plateau highlights the critical role of genomic tools in resolving taxonomic ambiguities and refining our understanding of Bharal biogeography and evolutionary history.

Conclusion

This study used microsatellite data to elucidate the genetic structure of *Pseudois nayaur* across the Indian Himalayan Region (IHR). Populations from the Union Territories of Ladakh, Himachal Pradesh, and Uttarakhand clustered within the western Himalayan (WH) lineage, indicating a shared evolutionary ancestry. In contrast, individuals from the Eastern Himalayas were genetically aligned with the Chinese Bharal (*P. n. szechuanensis*), highlighting a clear east–west genetic discontinuity.

Given the ecological, topographic, and climatic divergence between these regions, we propose recognising WH and EH populations as distinct Evolutionarily Significant Units (ESUs) and recommend their management as separate Management Units (MUs) for conservation. These findings clarify lineage boundaries within *P. nayaur*, enhance understanding of its evolutionary history, and provide a critical baseline for future conservation planning and population genetic monitoring in the IHR.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10709-025-00244-5.

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Author contributions Conceptualization: SKG, Sampling: DS, GS, PP, SL, Data curation: DS, Formal analysis: DS, GS, Resources: SKG, KV, Supervision: SKG, KV, SS, Writing original draft: DS, GS, Review & Editing: SKG, SL, KV, SS, Permission/Credit images, We used our images in this study. The sampling location and distribution maps were created in-house using the template of the Survey of India map.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare that they have received no financial support for this study.

Ethical approval All fieldwork was conducted in compliance with relevant national regulations. Prior approval for non-invasive sampling and surveys was obtained from the Forest Departments of Uttarakhand, Himachal Pradesh, Ladakh, and Sikkim.

Research involving human and animal participant As the study involved only non-invasive sample collection, approval for the research was obtained through appropriate permissions from the respective Forest Departments. Institutional Animal Ethics Committee (IAEC) clearance was not required.

References

Aryal A, Brunton DH, Campus A et al (2014) Human-Carnivore conflict: ecological and economical sustainability of predation on livestock by snow Leopard and other carnivores in the himalaya Human – carnivore conflict : ecological and economical sustainability of predation on livestock. https://doi.org/10.1007/s11625-014-0246-8. by snow leop

Benton TG, Bowler DE (2012) Linking dispersal to Spatial dynamics. Dispersal ecology and evolution. Oxford University Press, pp 251–265

Bhattacharya A, Chatterjee N, Rawat GS, Habib B (2020) Blue sheep resource selection in alpine grasslands of a Western Himalayan landscape – A point process approach. Zool Stud 59:1–15. https://doi.org/10.6620/ZS.2020.59-11

Chang YY, Chao MC, Ding ST et al (2012) Development of microsatellite markers in an ungulate mammal, the Formosan Serow (Capricornis swinhoei). Conserv Genet Resour 4:755–757. https://doi.org/10.1007/s12686-012-9637-2

Chetri M, Odden M, Wegge P (2017) Snow Leopard and Himalayan wolf: food habits and prey selection in the central himalayas, Nepal. PLoS ONE 12:e0170549. https://doi.org/10.1371/journal.pone.0170549

Dalui S, Khatri H, Singh SK et al (2020) Fine-scale landscape genetics unveiling contemporary asymmetric movement of red panda (Ailurus fulgens) in Kangchenjunga landscape, India. Sci Rep 10:1–13. https://doi.org/10.1038/s41598-020-72427-3

Dolker S, Jabin G, Singh SK et al (2023) Landscape genetics identified conservation priority areas for blue sheep (Pseudois nayaur) in the Indian Trans-Himalayan region. Sci Rep 13:18152. https://doi.org/10.1038/s41598-023-44823-y

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software<scp>structure: a simulation study. Mol Ecol 14:2611–2620. https://doi.org/10.1111/j.1365-294X.2005.02553.x

Frankham R (2012) How closely does genetic diversity in finite populations conform to predictions of neutral theory? Large deficits in regions of low recombination. Heredity (Edinb) 108:167–178. https://doi.org/10.1038/hdy.2011.66

Funk WC, Caminer M, Ron SR (2012) High levels of cryptic species diversity uncovered in Amazonian frogs. Proc R Soc B Biol Sci 279:1806–1814. https://doi.org/10.1098/rspb.2011.1653

Gao H, Nannan LI, Huang Y et al (2020) Taxonomic status of Chinese blue sheep (Pseudois nayaur): new evidence of a distinct subspecies. Integr Zool 15:202–212. https://doi.org/10.1111/1749-4877.12422

Gaur A, Singh A, Arunabala V, Umapathy G, Shailaja K, Singh L (2002) Development and characterization of 10 novel microsatellite markers from Chital deer (Cervus axis) and their cross–amplification in other related species. Mol Ecol Notes 3:607–609

Geist V (1987) On the evolution of the caprinae. The biology and management of capricornis and related mountain antelopes. Springer Netherlands, Dordrecht, pp 3–40

Gupta, SK, Kumar A, Hussain SA (2013) Extraction of PCR-amplifiable DNA from a variety of biological samples with uniform success rate. Conserv Genet Resour 5:215–217

Hassanin A, Delsuc F, Ropiquet A et al (2012) Pattern and timing of diversification of cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes. Comptes Rendus - Biol 335:32–50. https://doi.org/10.1016/j.crvi.2011.11.002

IUCN 2017 (2017) The IUCN Red List of Threatened Species

Jones KC, Levine KF, Banks JD (2002) Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (Cervus elaphus canadensis) Mol Ecol Notes 2:425–427

Joshi BD, Kumar V, De R et al (2022) Mitochondrial cytochrome b indicates the presence of two paraphyletic diverged lineages of the blue sheep pseudois Nayaur across the Indian himalaya: conservation implications. Mol Biol Rep 49:11177–11186. https://doi.org/10.1007/s11033-022-07832-0

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program < scp > cervus accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099–1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x

Lecis R, Pierpaoli M, Biro ZS et al (2006) Bayesian analyses of admixture in wild and domestic cats (Felis silvestris) using linked microsatellite loci. Mol Ecol 15:119–131. https://doi.org/10.1111/j.1365-294X.2005.02812.x

Li J, Fang X (1999) Uplift of the Tibetan plateau and environmental changes. Chin Sci Bull 44:2117–2124. https://doi.org/10.1007/BF03182692

Li M, Wei F, Goossens B et al (2005) Mitochondrial phylogeography and subspecific variation in the red panda (Ailurus fulgens): implications for conservation. Mol Phylogenet Evol 36:78–89. https://doi.org/10.1016/j.ympev.2004.12.016

Luenser K, Fickel J, Ludwig A (2005) Evolution of caprine and ovine β-defensin genes. Immunogenetics 57:487–498. https://doi.org/10.1007/s00251-005-0001-5

Lyngdoh S, Shrotriya S, Goyal SP et al (2014) Prey preferences of the snow Leopard (Panthera uncia): regional diet specificity holds global significance for conservation. PLoS ONE 9:e88349. https://doi.org/10.1371/journal.pone.0088349

Mishra C (1997) Livestock depredation by large carnivores in the Indian trans-Himalaya. Environ Conserv 24:338–343

Mishra C, Van Wieren SE, Ketner P et al (2004) Competition between domestic livestock and wild bharal pseudois Nayaur in the Indian Trans-Himalaya. J Appl Ecol 41:344–354. https://doi.org/10.1111/j.0021-8901.2004.00885.x

Mukesh, Sharma LK, Kumar VP et al (2013) Loss of genetic diversity and inbreeding in Kashmir red deer (Cervus Elaphus hanglu) of Dachigam National park, Jammu & kashmir, India. BMC Res Notes 6:326. https://doi.org/10.1186/1756-0500-6-326

Namgail T, Fox JL, Bhatnagar YV (2004) Habitat segregation between sympatric Tibetan argali Ovis Ammon hodgsoni and blue sheep pseudois Nayaur in the Indian Trans-Himalaya. J Zool 262:57–63. https://doi.org/10.1017/S0952836903004394

Oli MK (1996) Seasonal patterns in habitat use of blue sheep pseudois Nayaur (Artiodactyla, Bovidae) in Nepal. Mammalia 60:187–193. https://doi.org/10.1515/mamm.1996.60.2.187

Pal R, Panwar A, Goyal SP, Sathyakumar S (2022a) Changes in ecological conditions May influence intraguild competition: inferring interaction patterns of snow Leopard with co-predators. PeerJ 10:e14277. https://doi.org/10.7717/peerj.14277

Pal R, Sutherland C, Qureshi Q, Sathyakumar S (2022b) Landscape connectivity and population density of snow leopards across a multi-use landscape in Western himalaya. Anim Conserv 25:414–426. https://doi.org/10.1111/acv.12754

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. Bioinformatics 28:2537–2539. https://doi.org/10.1093/bioinformatics/bts460

Poetsch M, Seefeldt S, Maschke M, Lignitz E (2001) Analysis of microsatellite polymorphism in red deer, roe deer, and fallow deer: Possible employment in forensic applications. Foren Sci Int 116:1–8

Pritchard JK (2007) Documentation for structure software: Version 2. 2. Statistics (Ber)

Ram AK, Yadav NK, Kandel PN et al (2021) Tracking forest loss and fragmentation between 1930 and 2020 in Asian elephant (Elephas maximus) range in Nepal. Sci Rep 11:1–14. https://doi.org/10.1038/s41598-021-98327-8

Ranjitsinh MK (2017) A life with wildlife: from princely India to the present. haper collins

Saini D, Yadav P, Kolipakam V et al (2023) The complete mitogenome of blue sheep (Pseudois nayaur) from the Indian Himalayan region and its comparative phylogenetic relationship with other related species. Anim Gene 29:200155. https://doi.org/10.1016/j.angen.2023.200155

Saini D, Sonker G, Parab T et al (2025) Molecular taxonomy suggests presence of two distinct lineages of blue sheep (Pseudois nayaur) in Indian Himalayan region. Biochem Genet. https://doi.org/10.1007/s10528-024-11014-x

Shrestha R, Wegge P (2008) Habitat relationships between wild and domestic ungulates in Nepalese trans-Himalaya. J Arid Environ 72:914–925. https://doi.org/10.1016/j.jaridenv.2007.12.002

Shrestha B, Aihartza J, Kindlmann P (2018) Diet and prey selection by snow leopards in the Nepalese Himalayas. PLoS ONE 13:e0206310. https://doi.org/10.1371/journal.pone.0206310

Suryawanshi KR, Bhatnagar YV, Mishra C (2010) Why should a grazer browse? Livestock impact on winter resource use by bharal pseudois Nayaur. Oecologia 162:453–462. https://doi.org/10.1007/s00442-009-1467-x

Tan S, Zou D, Tang L et al (2012a) Molecular evidence for the subspecific differentiation of blue sheep (Pseudois nayaur) and polyphyletic origin of Dwarf blue sheep (Pseudois schaeferi). Genetica 140:159–167. https://doi.org/10.1007/s10709-012-9667-4

Tan S, Zou D, Tang L et al (2012b) Molecular evidence for the subspecific differentiation of blue sheep (Pseudois nayaur) and polyphyletic origin of Dwarf blue sheep (Pseudois schaeferi). Genetica 140:159–167. https://doi.org/10.1007/s10709-012-9667-4

Tan S, Wang Z, Jiang L et al (2017) Molecular phylogeny and phylogeography of genus pseudois (Bovidae, Cetartiodactyla): New insights into the contrasting phylogeographic structure. Ecol Evol 7:7047–7057. https://doi.org/10.1002/ece3.3269

Vaiman D, Osta R, Mercier D, Grohs C, Leveziel H (1992) Characterization of five new bovine dinucleotide repeats. Anim Genet 23:537–541

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538. https://doi.org/10.1111/j.1471-8286.2004.00684.x

Wan HY, Cushman SA, Ganey JL (2018) Habitat fragmentation reduces genetic diversity and connectivity of the Mexican spotted owl: A simulation study using empirical resistance models. Genes (Basel) 9. https://doi.org/10.3390/genes9080403

Wang X-M, Cao L-R, Liu Z-S, Fang S-G (2006) Mitochondrial DNA variation and matrilineal structure in blue sheep populations of

Helan mountain, China. Can J Zool 84:1431–1439. https://doi.org/10.1139/z06-137

Wilberg MJ, Dreher BP (2004)) <scp>genecap: a program for analysis of multilocus genotype data for non-invasive sampling and capture-recapture population Estimation. Mol Ecol Notes 4:783–785. https://doi.org/10.1111/j.1471-8286.2004.00797.x

Zeng B, Xu L, Yue B et al (2008) Molecular phylogeography and genetic differentiation of blue sheep pseudois Nayaur szechuanensis and pseudois schaeferi in China. Mol Phylogenet Evol 48:387–395. https://doi.org/10.1016/j.ympev.2008.05.010

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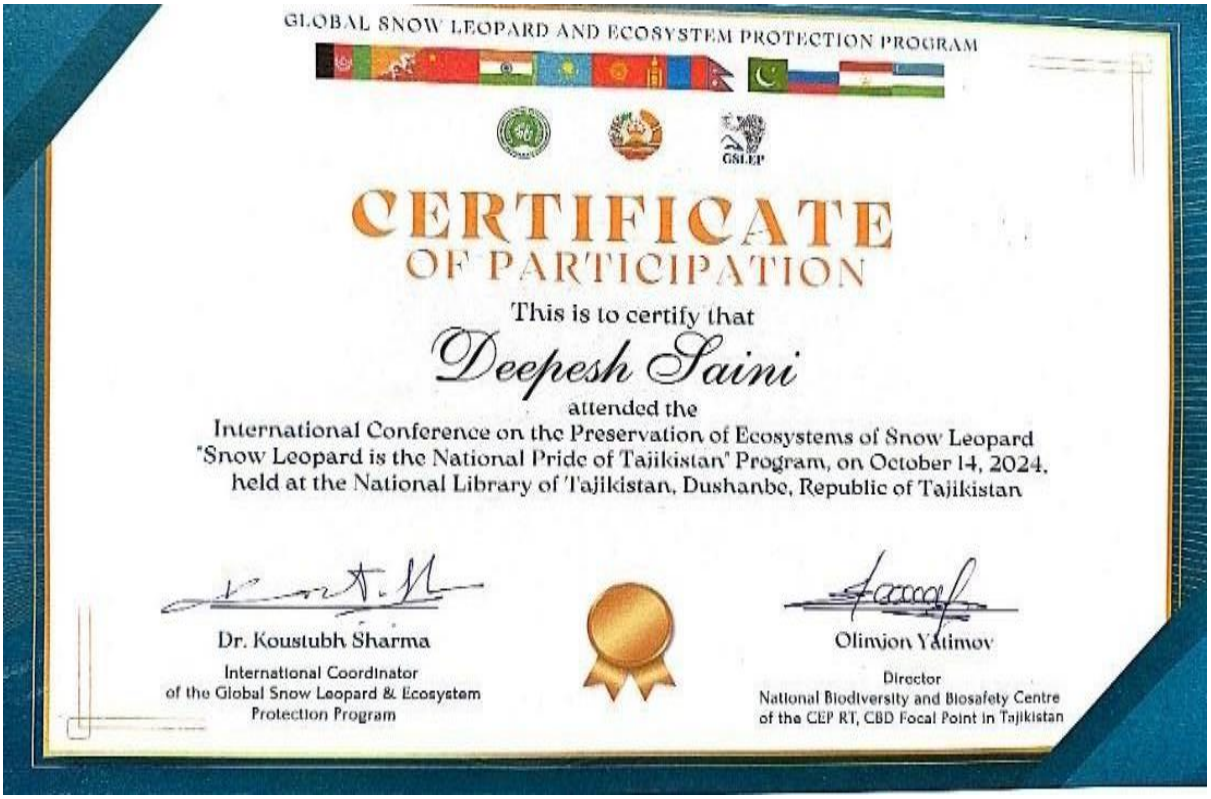
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