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RESEARCH ARTICLE

Genetic characterization of rare wild sheep in Uzbekistan based on mitochondrial DNA 16S rRNA genes

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Abstract

In this study, three rare wild sheep subspecies inhabiting Uzbekistan – *Ovis ammon severtzovi*, *Ovis vignei bochariensis*, and *Ovis vignei arkal* were genetically characterized using partial mitochondrial 16S rRNA gene sequences obtained from non-invasive samples. Molecular analysis confirmed the genetic identification of all three taxa within the genus *Ovis*. Comparative analysis of mitochondrial 16S rRNA gene sequences revealed low genetic divergence among the investigated taxa, ranging from 0.46% to 1.40%. Phylogenetic reconstruction supported the formation of well-supported monophyletic clades, with bootstrap values ranging from 92% to 97%, corresponding to the major urial and argali lineages. The Ustyurt and Bukhara sheep formed distinct subclades within the urial lineage, whereas the Kyzylkum sheep clustered with argali representatives. The results demonstrate that non-invasive genetic sampling provides a practical and minimally disruptive approach for the molecular identification of rare and endangered wild sheep. This method eliminates the need for direct animal capture or handling, thereby reducing stress and potential impacts on the study animals, while requiring relatively small amounts of biological material and limited labor and financial resources. Therefore,

non-invasive genetic sampling may be a valuable tool for monitoring population structure, genetic diversity, and distribution in rare and threatened wildlife species.

Keywords
Ovis, wild sheep, non-invasive method, mtDNA, PCR, phylogeny

Introduction

The family Bovidae is widely distributed across most continents and occupies a broad range of ecological niches, although it is naturally absent from Antarctica, Australia, and South America. The genus *Ovis* belongs to this family and includes several wild and domestic sheep species. Based on morphological characteristics, chromosome number, and geographical distribution, representatives of the genus *Ovis* are generally divided into seven groups: *Ovis ammon* L. 1758, *O. orientalis* Gmelin 1774, *O. vignei* Blyth 1841, *O. canadensis* Shaw 1804, *O. dalli* Nelson 1884, *O. nivicola* Eschscholtz 1829, and domestic sheep *Ovis aries* L. 1758 (Nadler et al. 1973; Rezaei et al. 2010; Upadhyay et al. 2021).

In Uzbekistan, representatives of the genus *Ovis* include the Kyzylkum argali (*Ovis ammon severtzovi* Nasonov, 1914), Ustyurt urial (*Ovis vignei arkal* Eversman, 1850), Bukhara urial (*Ovis vignei bochariensis* Nasonov, 1914), and Tien Shan argali (*Ovis ammon karelini* Severtzov, 1873). These taxa are considered rare and protected species within the Uzbekistan (Shernazarov et al. 2006). Historical records indicate that populations of Tien Shan argali were occasionally observed between 1979 and 1999 in the Ugam, Maidantal, and Talas Ala-Too mountain ranges, most likely as a result of seasonal migration from neighboring Kazakhstan (Mitropolskaya, 2009).

Molecular genetic approaches have significantly improved the understanding of the taxonomy and evolutionary history of wild sheep. Earlier studies based on mitochondrial DNA (mtDNA) control region sequences demonstrated that domestic sheep and mouflons belong to the same evolutionary lineage, whereas argali and urial sheep form genetically distinct groups (Hiendleder et al. 1998). Subsequent phylogenetic analyses using mtDNA cytochrome *b* sequences further confirmed that *O. orientalis* and *O. vignei* constitute separate monophyletic groups and supported the Asian origin of the genus *Ovis* (Rezaei et al. 2010).

Recent genomic studies have also clarified the taxonomic position of Uzbek wild sheep. Dotsev et al. (2023), using mitochondrial Cytb gene sequences, demonstrated that *Ovis ammon* severtzovi, previously considered a separate taxon, should be classified as a subspecies of *O. ammon*. Such findings emphasize the importance of molecular markers in resolving taxonomic ambiguities and understanding phylogenetic relationships among closely related wild sheep populations.

Mitochondrial DNA is widely used in vertebrate taxonomy, phylogenetics, and population genetics because of its relatively rapid evolutionary rate and maternal inheritance. Compared with nuclear DNA, mtDNA accumulates mutations more rap-

idly, making it especially useful for studying recent evolutionary and demographic processes (Bruford et al. 2003; Pesole et al. 1999; Kuchboev et al. 2024). Among mitochondrial genes, the 16S rRNA and cytochrome b regions are commonly applied in phylogenetic and molecular identification studies of ungulates and other mammals.

In recent years, non-invasive genetic sampling has become an effective approach for studying rare and endangered wildlife species. The collection of feces, hair, or other biological traces enables researchers to obtain genetic information without disturbing animals in their natural habitats. Compared with traditional capture-based methods, non-invasive sampling is less labor-intensive, more cost-effective, and suitable for long-term biodiversity monitoring programs (Waits and Paetkau 2005).

Therefore, the present study aimed to investigate the molecular genetic characteristics of endemic wild sheep subspecies in Uzbekistan using partial nucleotide sequences of the mtDNA 16S gene obtained from non-invasive samples.

Materials and methods

Sample Collection

Non-invasive genetic samples, including feces and hair, were collected from three endemic wild sheep subspecies inhabiting Uzbekistan: *Ovis ammon severtzovi* from the Nuratau State Reserve, *Ovis vignei bochariensis* from the Jeyran Ecological Center, and *Ovis vignei arkal* from the The National Natural Park "Janyubiy Ustyurt" (Table 1, Fig.1).

Table 1. Genetic variability and geographic distribution of *Ovis* species

Species	Nuratau State Reserve	The National Natural Park "Janyubiy Ustyurt"	Jeyran Ecological Center	
	Feces	Feces	Feces	Hair
Ovis ammon severtzovi Nasonov, 1914	+	–	–	–
Ovis vignei bochariensis Eversman, 1850	–	–	+	+
Ovis vignei arkal Nasonov, 1914	–	+	–	–

A total of 26 fecal samples representing all described wild sheep taxa occurring in Uzbekistan were collected, including *O. ammon severtzovi* (n = 11), *O. vignei bochariensis* (n = 9), and *O. vignei arkal* (n = 6). Sampling was conducted along predetermined transects crossing areas with high wild sheep densities. Fecal samples were collected from locations known to support resident populations, including animal trails and mountain gorges within the surveyed protected areas. Each

sample was placed in a sterile vial containing granular silica gel and stored at room temperature until DNA extraction. In addition, three hair samples of Bukhara urial (*O. vignei bochariensis*), maintained at the Jeyran Ecological Center, were collected during the expeditions and preserved in silica gel to minimize DNA degradation.

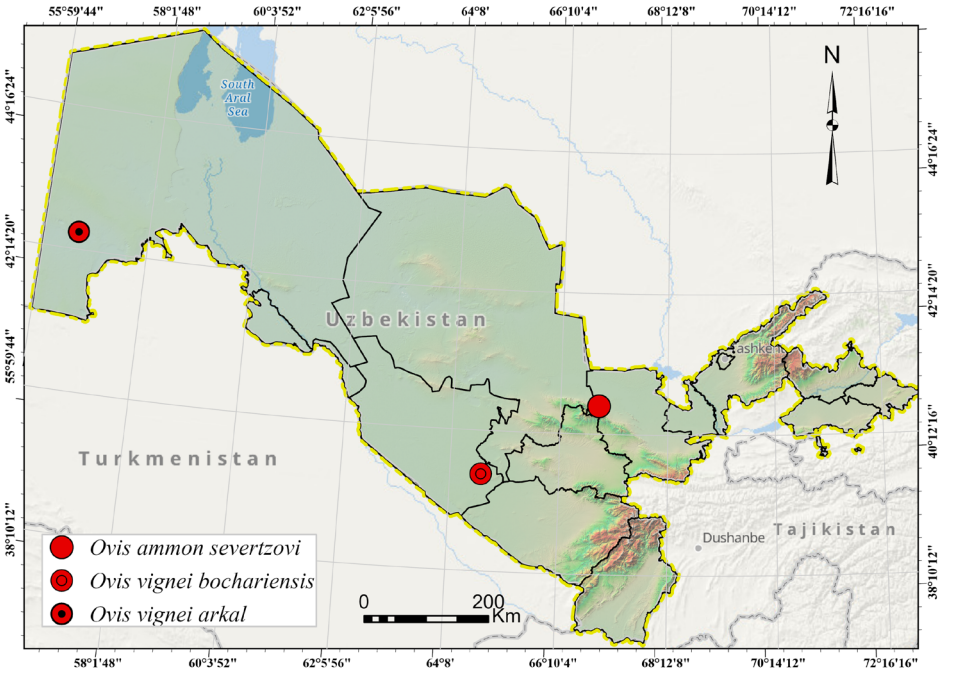

Figure 1. Geographic locations of the *Ovis* genetic samples used for molecular analyses. Samples were collected from the Nuratau State Reserve (*O. ammon severtzovi*), the Jeyran Ecological Center (*O. vignei bochariensis*), and the Southern Ustyurt National Nature Park (*O. vignei arkal*).

Fecal samples were collected, whenever possible, immediately after defecation, whereas hair samples were collected directly from the animals. Disposable sterile materials were used during sample collection, and separate collection instruments were used for each sample to minimize cross-contamination. Fecal samples were collected using sterile paper funnels, individually labelled, and stored accordingly. Hair samples were placed dry in sterile, individually labelled tubes. The collected samples were transported to the laboratory at +4 °C, and DNA extraction was performed within 3 days of sample collection.

To maximize the recovery of host DNA, only the outer surface of each fecal pellet, which contains sloughed intestinal epithelial cells, was sampled for DNA extraction. This approach increases the proportion of endogenous host DNA while reducing contamination from environmental microorganisms and dietary material. Approximately 0.8–1.2 g of fecal material was used for each extraction, following the recommendations of the DNA extraction kit manufacturer.

DNA Extraction

Genomic DNA was extracted from collected fecal and hair samples using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Extracted DNA samples were stored at –20°C until further molecular analyses. The concentration and quality of the extracted DNA were assessed using a NanoDrop Plus spectrophotometer (Thermo Fisher Scientific, USA). Of the 26 collected samples, 12 yielded DNA of sufficient quality for subsequent molecular genetic analyses, corresponding to an extraction success rate of 19.1% (Table 2).

Table 2. DNA concentration isolated from wild sheep subspecies

Sheep subspecies	DNA amount (ng/µl)	DNA amount sequenced (ng/µl)
Ovis ammon severtzovi	5.9–14.3	2.4–4.9
Ovis vignei arkal	6.1–17.6	1.8–21.3
Ovis vignei bochariensis	5.3–11.7	1.9–9.8

PCR amplification of mtDNA 16S rRNA Gene

Partial fragments of the mitochondrial 16S rRNA gene were amplified using the primer pair 16S cp-F (5′-CGAGGGCTTTACTGTCTCTT-3′) and 16S cp-R (5′-CCTATTGTCGATATGGACTCT-3′) described by Caragiulo et al. (2013). The PCR reaction was prepared using a reagent set from Silex (Russia), comprising 10x PCR buffer, dNTP solution, Taq polymerase, and sterile water. The amplification cycle was conducted on a ProFlex PCR system (ThermoFisher). Stages 2 through 4 were repeated for 30 cycles (Kuchboev and Krücken 2022).

PCR amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 94°C for 5 min; followed by 35 cycles consisting of denaturation at 95°C for 45 s, primer annealing at 55°C for 45 s, and extension at 72°C for 1 min 40 s; with a final extension step at 72°C for 5 min.

Amplified PCR products were analyzed by agarose gel electrophoresis. Genomic DNA samples were separated on 2% agarose gels, whereas PCR products were analyzed using 1.5% agarose gels. PCR products were purified using the Biospin Gel Extraction Kit prior to sequencing.

DNA sequencing and phylogenetic analyses

Sequencing of the amplified mtDNA 16S rRNA gene fragments was performed using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit on a SeqStudio Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA) at the Center for Advanced Technologies, Ministry of Innovative Development of the Republic of Uzbekistan.

Genetic distances among the studied Ovis taxa were calculated using the Kimura 2-Parameter (K2P) model (Kimura 1980). The resulting chromatograms (in ab1 format) were processed using the Chromas 2.6.6 program (Technelysium Ltd., South Brisbane, Australia, 2018) and converted into FASTA format. The analysis of nucleotide sequences was conducted using the BLAST algorithm, facilitating the identification of closely related species of wild sheep within the studied forms. Data selection for analysis included all relevant forms within the compared species as well as nucleotide sequences from representatives of different genera for use as an "outgroup." Phylogenetic trees were generated using MEGAX, with a maximum-likelihood analysis performed on the IQ-TREE server (version 1.6.12, Nguyen et al. 2015). The selection of suitable models for this analysis was conducted using the MEGAX package.

For phylogenetic analysis, the new sequences were aligned with wild sheep sequences of the mitochondrial 16S rRNA gene region published in GenBank, with two *Capra falconeri* Wagner, 1839 sequences (OP722695 and OR799853) used as outgroups. The final tree visualization and editing were carried out using iTOL v6.6.

Results

Molecular characterization of wild sheep subspecies in Uzbekistan

Partial nucleotide sequences of the mitochondrial 16S rRNA gene were successfully amplified and sequenced from non-invasive genetic samples of *O. ammon severtzovi*, *O. vignei bochariensis*, and *O. vignei arkal*. Approximately 216 bp fragments were obtained and analyzed for comparative molecular characterization.

Comparative analysis of mtDNA 16S rRNA gene sequences revealed a low level of genetic divergence among the investigated taxa, ranging from 0.46 % to 1.4 % using oh the Kimura 2-Parametr model (Fig. 2).

The genetic distance analysis revealed no detectable genetic divergence between the two *O. a. severtzovi* specimens from Uzbekistan (UZB) and the reference sequence OQ513283 retrieved from GenBank. The K2P genetic distance was 0.0 %, with no nucleotide differences detected between the sequences. This result indicates that the specimens were identical across the analyzed genetic marker and exhibited no detectable intraspecific variation at this locus.

A similar pattern was observed for *O. v. arkal*. The K2P distance between the UZB specimen and the GenBank reference sequence OQ513287 was 0.0%, with zero nucleotide differences. Thus, the analyzed *O. v. arkal* sequences were identical across the studied fragment. In contrast, comparison between *O. v. arkal* and *O. a. severtzovi* revealed a K2P distance of 1.40%, corresponding to three nucleotide differences. This divergence indicates detectable genetic differentiation between these two taxonomic groups at the analyzed mitochondrial marker.

No genetic differences were detected between the *O. v. bochariensis* UZB specimen and the GenBank reference sequence PQ652212. The K2P distance was 0.0%, and no nucleotide differences were observed. This result suggests a high degree of sequence conservation within *O. v. bochariensis* at the analyzed locus. In comparison, the genetic distance between *O. v. bochariensis* and *O. v. arkal* was 0.46%, corresponding to a single nucleotide difference. Thus, the genetic divergence between these two taxa was considerably lower than that observed between *O. a. severtzovi* and *O. v. arkal*.

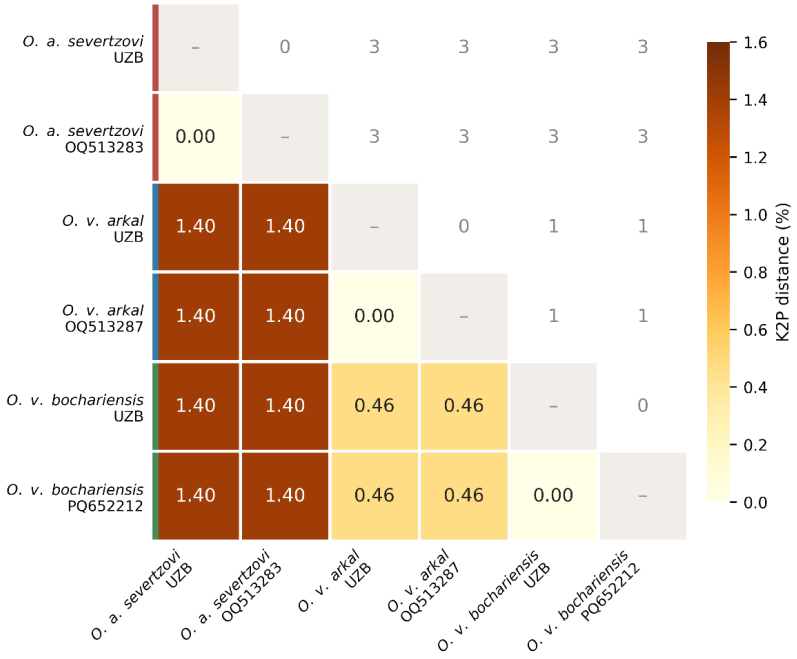

Figure 2. Genetic distance matrix among the studied *Ovis* subspecies. Note: The lower triangular matrix presents K2P genetic distances as percentages, whereas the upper triangular matrix shows the corresponding numbers of nucleotide differences.

Overall, the genetic distance analysis demonstrated very low sequence variation within the studied taxonomic groups, whereas measurable differentiation was observed among groups. The 0.0% K2P distances between the Uzbek specimens and their corresponding GenBank reference sequences indicate complete sequence identity at the analyzed marker. Among the intergroup comparisons, the highest genetic distance was observed between *O. a. severtzovi* and *O. v. arkal* (1.40%; three nucleotide differences), whereas the lowest was recorded between *O. v. arkal* and *O. v. bochariensis* (0.46%; one nucleotide difference).

Taken together, the K2P analysis indicates a certain degree of genetic differentiation among *O. a. severtzovi*, *O. v. arkal*, and *O. v. bochariensis*, despite the low sequence variability within each group. The particularly small genetic distance between *O. v. arkal* and *O. v. bochariensis* suggests a closer genetic relationship between these taxa based on the analyzed molecular marker. However, these results should be interpreted cautiously, as genetic distances based on a single marker do not by themselves establish phylogenetic relationships or taxonomic status.

Phylogenetic analysis

Phylogenetic reconstruction based on partial mtDNA 16S rRNA sequences demonstrated that all analyzed representatives of the genus Ovis formed a supported monophyletic group using Maximum Likelihood (ML) method.

The ML phylogenetic tree constructed using the Tamura–Nei model showed strong bootstrap support values ranging from 92% to 97% (Fig. 3). Two principal phylogenetic lineages were identified. The first lineage included urial representatives (*O. vignei*, *O. vignei arkal*, and *O. vignei bochariensis*) originating from Uzbekistan (Uzbek 1, 2), Iran, and Tajikistan. The second lineage comprised argali representatives, including *O. ammon* and *O. ammon severtzovi*.

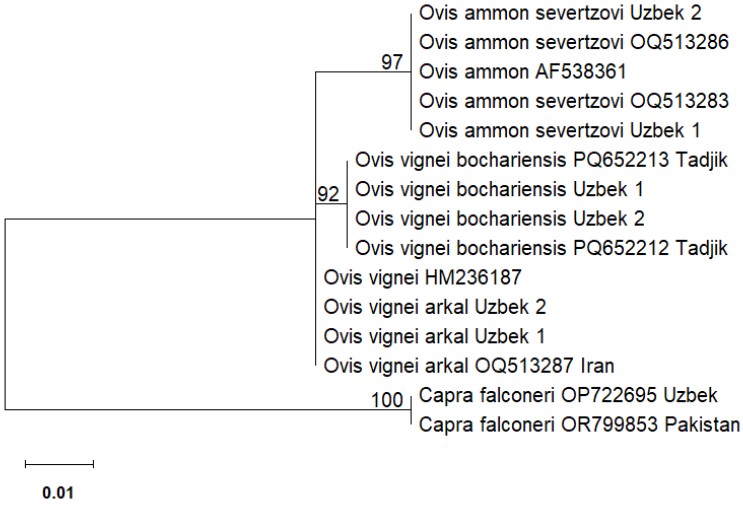

Figure 3. Maximum Likelihood phylogenetic tree of wild sheep species of the genus *Ovis* based on partial mtDNA 16S rRNA gene sequences constructed using the Tamura–Nei model.

The obtained mtDNA 16S rRNA partial nucleotides result confirms the phylogenetic position of *O. ammon severtzovi* within the argali clade and its classification as a subspecies of *O. ammon*. This has been previously recognized by other scientists

(Rezaei et al. 2010). In contrast, the Ustyurt and Bukhara wild sheep populations formed distinct monophyletic subgroups within the urial lineage. *Capra falconeri* was used as an outgroup species in the phylogenetic analyses.

The nucleotide sequences generated in this study have been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under the following accession numbers: OL907312 and PZ834801 for *Ovis ammon severtzovi*; OL907313 and PZ834804 for *Ovis vignei arkal*; and OR240284 and PZ834803 for *Ovis vignei bochariensis*.

Discussion

The application of non-invasive genetic sampling methods also represents a significant advantage of this study. Collection of fecal and hair samples minimizes stress and disturbance to rare and protected wildlife species while enabling effective molecular analyses (Quasim et al. 2018; Ferreira et al. 2018; Kuchboev et al. 2017, 2020; Sobirov et al. 2025). This approach is especially important for endangered ungulates inhabiting remote or protected ecosystems where direct capture and handling are difficult or ethically undesirable.

Complementing traditional methods such as trapping and telemetry (Furnas et al. 2018; Newediuk et al. 2022), fecal DNA sampling provides valuable information on population dynamics, genetic diversity, and ecosystem interactions while minimizing disturbance and stress to wildlife. Previous studies have demonstrated that methods for the collection and preservation of fecal samples, including ethanol-based preservation of fecal pellets, are effective for subsequent DNA extraction and genetic analyses (Lounsberry et al. 2015; Kierepka et al. 2016). Fecal samples offer several advantages over other non-invasive sampling methods because they are readily detectable in the field and generally contain sufficient quantities of host-derived genetic material. These characteristics facilitate reliable molecular analyses that can provide important insights into species biology, population structure, and conservation status (Ramón-Laca et al. 2015).

Our results further demonstrate the effectiveness of non-invasive genetic sampling for monitoring *Ovis* species. The application of fecal DNA analysis can complement conventional field-based approaches and provide a practical, low-disturbance means of obtaining genetic information from wild populations. We therefore recommend the broader integration of molecular genetic sampling into population monitoring programs, particularly for rare, threatened, and difficult-to-capture species. Such approaches may substantially improve population assessment and contribute to more effective conservation and management strategies.

The taxonomy and evolutionary relationships within the genus *Ovis* have long remained controversial because morphological characteristics alone are often insufficient for accurate species delimitation. Traits such as horn morphology, coat coloration, and body size may vary considerably under environmental influences and

do not always reflect phylogenetic history (Valdez et al. 1978). Therefore, molecular genetic approaches have become essential tools for resolving taxonomic uncertainties within the family Bovidae.

In the present study, partial mtDNA 16S rRNA gene sequences demonstrated genetic similarity among the investigated wild sheep taxa of Uzbekistan, with genetic divergence ranging from 0.46% to 1.40%. These relatively low divergence values suggest recent evolutionary differentiation among the analyzed taxa and are consistent with previous phylogenetic studies of the genus *Ovis* based on mitochondrial markers (Hiendleder et al. 1998; Rezaei et al. 2010).

Phylogenetic reconstruction clearly separated the analyzed taxa into two major evolutionary lineages corresponding to urial and argali groups. The clustering of *Ovis ammon severtzovi* within the argali clade supports the conclusions of Dotsev et al. (2023), who proposed that this taxon should be classified as a subspecies of *O. ammon* rather than an independent species. At the same time, the formation of distinct monophyletic subclades by *O. vignei arkal* and *O. vignei bochariensis* indicates a certain degree of genetic differentiation within the urial lineage.

Mitochondrial DNA markers, particularly the 16S rRNA and cytochrome b regions, have been widely applied in studies of mammalian phylogeny, population genetics, and molecular taxonomy because of their relatively rapid mutation rates and maternal inheritance patterns (Bruford et al. 2003; Pesole et al. 1999). Previous studies have shown that mtDNA markers provide valuable insights into the evolutionary history and demographic structure of wild sheep populations (Pedrosa et al. 2005; Pidancier et al. 2006). The present study further confirms the effectiveness of these markers for identifying endemic wild sheep taxa in Central Asia.

Overall, the obtained results contribute to a better understanding of the phylogenetic relationships and molecular taxonomy of wild sheep in Uzbekistan and provide practical molecular tools for their reliable identification and conservation management.

Conclusion

This study provides molecular genetic data on endemic wild sheep subspecies inhabiting Uzbekistan based on partial nucleotide sequences of the mitochondrial 16S rRNA gene obtained using a non-invasive genetic sampling approach. Comparative analysis of the partial 16S rRNA gene sequences revealed low levels of genetic divergence among the studied taxa, ranging from 0.46% to 1.40%, based on the Kimura 2-Parameter (K2P) model. Phylogenetic reconstruction nevertheless supported the presence of two major evolutionary lineages corresponding to argali and urial sheep. *Ovis ammon severtzovi* was placed within the argali lineage, whereas *Ovis vignei arkal* and *Ovis vignei bochariensis* formed distinct monophyletic groups within the urial clade.

These findings demonstrate that mitochondrial DNA markers, combined with non-invasive genetic sampling, provide an effective approach for molecular identification, phylogenetic assessment, and conservation monitoring of rare wild sheep populations. The molecular markers evaluated in this study may serve as useful tools for biodiversity conservation, population management, ecological monitoring, and anti-poaching efforts in Uzbekistan and neighboring regions.

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