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RESEARCH ARTICLE

Species identification based on the fecal DNA samples of the Caprinae

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Abstract

Fecal analysis is a useful tool for studying the species identity of rare mammals. The possibility of using non-invasive biological materials in molecular genetic studies of rare bovids is shown, using the example of the markhor and Siberian ibex of Uzbekistan. Field work including noninvasive genetic sampling collection was carried out in the study area in spring and autumn 2022-2023 in the Hissar, Surkhan State Reserves and Ugam-Chatkal State National Natural Park and Termez zoo in Uzbekistan. We used species-specific 16S rRNA mitochondrial gene fragments for polymerase chain reaction amplification for species identification. The results of the molecular analysis with the 16S rRNA mitochondrial gene allowed the identification of *Capra sibirica*, *C. falconeri* and *C. hircus* belonging to the subfamily Caprinae using a noninvasive genetic sampling method. This method is quite easy to use, while avoiding direct contact with the animal, which minimizes the degree of impact on the object being studied and does not require significant material and labor costs for researchers. We believe that noninvasive genetic sampling is emerging as one of the most effective and accurate methods for estimating the population size of animals, and we recommend considering this approach for endangered and rare species. The protocol developed could be a valuable tool in the management and conservation of the *Capra* species occurring on the Uzbekistan.

Keywords
Capra, noninvasive genetic sampling, PCR, rDNA, 16S, species identification

Introduction

The concept of biological diversity occupies an important place in modern science and environmental policy. In recent decades, the natural resources of our planet have been constantly declining. The reasons for this are chemical pollution of the environment, destruction of existing habitats of wild species, intentional or accidental introduction of invasive species into nature, as well as the results of human economic activity along with climate change. Despite the consolidated efforts of the countries party to the Convention on Biological Diversity, the problem has not yet been resolved (Voronova and Levykina 2019).

The main representatives of wild ruminants are species of the bovid family (Bovidae). Currently, the bovid family includes 143 species, which is almost 55% of ungulates. There are 15 species found in the Commonwealth of independent states countries (Sobirov et al. 2022). In Uzbekistan, there are 6 species and subspecies of bovid animals: *Capra sibirica* (Pallas, 1776), *C. falconeri* (Wagner, 1839), *Ovis vignei* Blyth, 1841 (*O. v. arcal* Eversmann, 1850, *O. v. bochariensis* Nasonov, 1914), *Ovis ammon* Linnaeus 1758 (*O. a. severtzovi* Nasonov, 1914, *O. a. karelini* Severtzov, 1873), *Saiga tatarica* Linnaeus 1766, *Gazella subgutturosa* (Güldenstädt, 1780), from of which 5 species are listed in the Red Book of the Republic of Uzbekistan and the IUCN Red List (Sobirov et al. 2022).

The Siberian ibex (*Capra sibirica*) inhabits rocky slopes and alpine meadows. It spends both summer and winter here, descending into the zone of juniper forests only in case of extreme lack of food and feeding mainly on herbaceous vegetation. It lives in separate herds ranging from tens to several hundred animals. The Siberian ibex is valued as a hunting and commercial animal. The hunt for him is carried out under special permission. The markhor (*C. falconeri*), like other goats, is an inhabitant of rocky mountain. In its way of life, it is in many ways similar to the Siberian ibex. It is a rare protected species and is listed in the Red Book of Uzbekistan (Sobirov et al. 2022). As noted, in addition to the listed wild representatives of goats, subfamily Caprinae includes domestic goats (*C. hircus*) bred in Uzbekistan, common in various landscapes. Since these animals are common in the mountain, steppe and desert regions of Uzbekistan, where they feed on pasture grasses and act as the main hosts of many helminthes, which are important components of ecosystems (Kuchboev et al. 2015).

Noninvasive genetic sampling is a relatively new approach to data collection that has great potential for wildlife zoologists. By extracting genetic material from feces, hair, feathers, or other sources of DNA, zoologists can collect important data about wild animal populations without having to capture or even observe individual animals (Bach et al. 2022). This approach has several advantages over traditional

sample collection methods. Non-invasive genetic sampling, compared to traditional methods of searching and capturing animals, usually does not require significant material and labor costs and allows one to obtain a sufficient number of biological samples for laboratory analysis.

Noninvasive genetic sampling has been introduced as a method for obtaining genetic samples from rare and elusive brown bears in Europe (Ferreira et al. 2018). Next, individual animals were identified using this method. Examples: Hair snares for grizzlies (Phoebus et al. 2020) and Himalayan wolf in Nepal (Werhahn et al. 2015); Pallas cat and wild boar identified by scat in mountain Tien Shan in Kirgizstan (McCarthy et al. 2010).

Therefore, as part of a study of the genetic diversity and population structure of wild goats in the reserves of Uzbekistan was in order to develop recommendations for a molecular inventory of animals using non-invasive collection of biomaterials. All this necessitates monitoring the population of species, a comprehensive study of its biology and ecology for scientifically based population management. The methods of molecular biology, which are now widely and universally used in population studies, have acquired an important role in these studies.

The purpose of this research study is to carry out the molecular identification of the species *Capra sibirica*, *C. falconeri* and *C. hircus* belonging to the subfamily Caprinae, using a non-invasive genetic sampling method in Uzbekistan.

Materials and methods

Genetic materials for molecular studies were collected between October 22, 2022, and March 20, 2023. Samples were obtained from *Capra sibirica* and *C. falconeri* in various protected areas including the Hissar State Reserve, Surkhan State Reserve, and Ugam-Chatkal National Park, all of which are managed by the State Committee of Ecology and Environmental Protection of the Republic of Uzbekistan. Additionally, samples from domestic goats (*Capra hircus*) and *C. falconeri* were collected at the Termez Zoo in the city of Termez (Table 1, Fig.1).

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

Species	Hissar Nature Reserve	Ugam-Chatkal National Park	Surkhan Nature Reserve	Termez Zoo
	Feces	Feces	Feces	Feces
Capra falconeri (Wagner, 1839)	–	–	+	+
C. sibirica (Pallas, 1776)	+	+	–	–
C. hircus Linnaeus 1758	–	–	–	+

Figure 1. Geographic locations of Caprinae samples utilized for molecular genetic analyses. Samples were collected from Ugam-Chatkal National Park, Hissar, Surkhan Reservoirs, and Termez Zoo.

Fecal samples were collected along predetermined routes that intersected areas densely populated by *C. sibirica* and *C. falconeri*. This collection targeted locations known for high concentrations of these goats, specifically along trails and in gorge mountain zones within the aforementioned reserves. For the preservation of collected biomaterials, tubes containing granulated silica gel were utilized. The samples were maintained at room temperature until DNA extraction. In total, three expeditions yielded 34 samples of wild goat feces and 10 samples of domestic goat feces, all stored in silica gel to prevent degradation.

To ensure adequate recovery of host DNA, only the outer surface of the fecal samples, which is in direct contact with the animals' epithelial membranes, was retained. Trimming away the outer layer maximized the isolation of host DNA while minimizing potential contamination from exogenous sources. Most DNA extraction kits typically require between 0.80 g and 1.20 g of fecal material for optimal performance. DNA isolation was performed using several extraction kits according to the manufacturers' protocols. The PereLink™ Genomic DNA Kit (Invitrogen, USA) provided superior results for fecal samples. All extractions were conducted on a dedicated laboratory bench and in a pre-polymerase chain reaction (PCR) laboratory to mitigate contamination risks.

The concentration and quality of the extracted genomic DNA were assessed using a NanoDrop One spectrophotometer (Thermo Scientific™). The quality was determined through measurement of the absorption wavelength ratio at 260 nm/280 nm, following the manufacturer's guidelines. Samples exhibiting ratios within the optimal range of 1.7 to 2.0 were further processed and diluted to a standard concentration of 10 ng/μl for downstream applications.

For the amplification of genomic DNA, PCR was performed using nucleotide primers (F-5' CGAGGGCTTTACTGTCTCTT -3'; R-5' CCTATTGTCGATATG-GACTCT -3') targeting mitochondrial 16S rRNA gene fragments. 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) following this protocol:

1. Initial DNA denaturation at 94 °C for 5 minutes;
2. Subsequent cycles of DNA denaturation at 94 °C for 45 seconds;
3. Primer annealing at 52 °C for 45 seconds;
4. Extension at 72 °C for 45 seconds;
5. Final extension at 68 °C for 15 minutes.

Stages 2 through 4 were repeated for 30 cycles (Kuchboev and Krücken 2022).

To visualize the PCR products, electrophoresis was performed on a 2% agarose gel. PCR products (25 µl volume) were loaded into the gel wells, and separation of DNA fragments was conducted at a voltage of 80 volts/cm on a 15 cm horizontal gel. A molecular weight marker (GeneRuler™ 100 bp DNA Ladder) was included in the outermost well to estimate fragment sizes.

For sequencing, PCR products were amplified in both directions with the same primers utilized for amplification, at the Center of Advanced Technologies of the Ministry of Higher Education, Science and Innovation of the Republic of Uzbekistan in Tashkent. 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 parasitic organisms 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.

The nucleotide sequence of the mitochondrial 16S rRNA gene region for *Gazella subgutturosa* (Güldenstädt, 1780) was included as an outgroup for the consensus tree analysis. The final tree visualization and editing were carried out using iTOL v6.6.

Results

We obtained a nucleotide sequence comprising 300 paired nucleotides from the 16S rRNA mitochondrial gene of genetic samples isolated from three goat species: *Capra sibirica*, *Capra falconeri*, and *Capra hircus*. Each sample was analyzed for species identification using species-specific primers designed to amplify regions of the 16S gene (Table 2).

Table 2. Matrix comparison of nucleotide sequences of 16S rRNA mitochondrial gene among *Capra* species

№	Species	1	2	3	4	5	6
1	*C. hircus* Uzb	–	0.06	2.3	2.7	2.7	2.7
2	*C. hircus* MH229952	2	–	3.0	3.4	3.4	3.4
3	*C. falconeri* Uzb	7	9	–	0.3	1.0	1.7
4	*C. falconeri* OP722695	8	10	1	–	1.3	1.3
5	*C. sibirica* Uzb	8	10	3	4	–	0.6
6	*C. sibirica* AF400655	8	10	5	4	2	–

As we revealed, the sequence comparison revealed that *C. hircus* Uzb differed from the GenBank sample of *C. hircus* (MH229952) by two nucleotides (0.06%). Further analysis showed a nucleotide difference of seven nucleotides (2.3%) between the domestic goat and *C. falconeri* Uzb, and an eight-nucleotide difference (2.7%) with *C. sibirica* Uzb. Between the species *C. falconeri* and other members of the genus *Capra*, differences were also apparent. Specifically, *C. falconeri* (OP722695) exhibited a difference of one nucleotide (0.3%) compared to its counterpart, while differences of eight nucleotides (2.3%) with *C. hircus* and three nucleotides (1%) with *C. sibirica* were noted. Additionally, differences in nucleotide sequences were identified among *C. sibirica*, *C. hircus*, and *C. falconeri* (Table 2).

Based on the analysis of the 16S rRNA gene sequences from *C. sibirica*, *C. falconeri*, and *C. hircus*, along with corresponding sequences from the GenBank database, we investigated the phylogenetic relationships of nine goat species (Fig. 2). Results indicated that the *C. falconeri* specimens identified in Uzbekistan exhibited 98–99% similarity to the subspecies *C. falconeri* (OP722695, OW568856, and NC020622). Similarly, *C. sibirica* Uzb demonstrated 98% similarity with bootstrap support when compared to samples *C. sibirica* (OW568913) and *C. sibirica* (AF400655). Notably, *C. hircus* samples showed 100% consistency (Fig. 2).

It is noteworthy that *C. ibex* (3) and *C. pyrenaica* (4) clustered together, supporting previous findings by Heptner (1988). Studies on the mtDNA of *Capra* species indicate that the Siberian ibex and Nubian ibex are distinct species, not closely related to the Alpine ibex (*C. ibex*). Furthermore, results show that Alpine goats and Pyrenean goats (*C. pyrenaica*) comprise a separate subgroup, distinct from the other species analyzed. The data obtained underscores the genetic isolation of *Capra* species and highlights the importance of environmental factors in shaping their evolutionary trajectories.

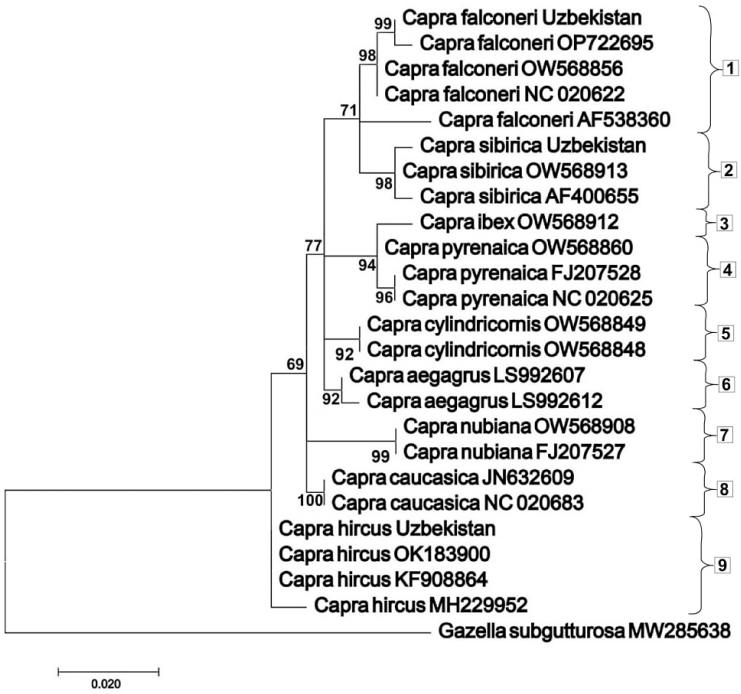

Figure 2. Phylogenetic tree of *Capra* species derived from 16S rRNA mitochondrial gene analysis using the Maximum Likelihood method.

Discussion

This study confirms the findings of Hammer et al. (2016), which demonstrated the separation of two subspecies: the Bukhara markhor (*C. falconeri heptneri*) and the markhor (*C. falconeri megaceros*), through the analysis of mitochondrial DNA COI genes. The average intragroup genetic distance was notably maximized within *C. sibirica*, indicating a potential for significant genetic variability within this species.

The phylogenetic tree we constructed reveals a distinct grouping of Caucasian goats (*C. caucasica*, *C. aegagrus*, *C. cylindricornis*), Nubian goats (*C. nubiana*), and the domestic goat (*C. hircus*), underlining their evolutionary relationships and divergence from other wild ungulates. Wild goats (*Capra* Linnaeus, 1758) inhabit mountainous regions of the southern Palearctic and can be further classified into eight species as identified by V.I. Sokolov (1959). Understanding the distribution and genetic variance of these species is crucial for their management and conservation.

The Siberian Mountain goat (*C. sibirica*) occupies a broad geographical range extending from Central and northeastern Afghanistan to regions in China, India, Kazakhstan, Kyrgyzstan, Mongolia, Pakistan, Russia, Tajikistan, and Uzbekistan (Reading et al. 2020). Meanwhile, populations of the markhor (*C. falconeri*) are

sustained around the southern border regions of Tajikistan, Afghanistan, Pakistan, Turkmenistan, Uzbekistan, and northern India. Such wide-ranging habitats highlight the necessity for localized conservation strategies tailored to specific ecological requirements and human impacts.

The application of fecal DNA collection as a methodological tool represents a significant advancement in non-invasive wildlife monitoring. Several studies (Kurose et al. 2005; Canu et al. 2017; Quasim et al. 2018; Ferreira et al. 2018) have championed this approach as a promising way to manage the conservation of elusive or endangered species. By complementing traditional methods such as trapping and telemetry (Furnas et al. 2018; Newediuk et al. 2022), fecal DNA sampling provides vital insights into population dynamics, genetics, and ecosystem interactions while minimizing stress on wildlife.

Research indicates that methods for collecting and extracting fecal DNA, such as the storage of pellets in ethanol (Lounsberry et al. 2015; Kierepka et al. 2016), are both effective and efficient. Fecal samples, in particular, have advantages over other non-invasive sampling methods, as they are conspicuous in the field and typically retain higher quantities of genetic material. This allows for robust analyses, which are critical for understanding the biology and conservation of the species concerned (Ramón-Laca et al. 2015).

Our findings reinforce the efficacy of non-invasive genetic sampling in monitoring *Capra* species, suggesting a shift towards molecular approaches for population assessment. The recommendations drawn from this study advocate for the increased use of genetic sampling techniques as reliable tools for estimating animal population sizes, especially for endangered and rare species.

Conclusions

Molecular analysis using the 16S rRNA mitochondrial gene facilitates the identification of genetically distinct species within the genus *Capra*, including *Capra sibirica*, *C. falconeri*, and *C. hircus*. Given the demonstrated success of this method, future research should leverage longer genetic sequences and analyze multiple specimens across geographical ranges to deepen our understanding of *Capra* phylogeny. Furthermore, it is imperative to incorporate nuclear DNA analysis to elucidate the evolutionary relationships within this genus comprehensively. The nucleotide sequences we generated for these conservation-critical species have been deposited in GenBank (NCBI) under the accessions *C. falconeri* – OP722695 and *C. sibirica* – OR234744, thereby enriching the genetic repository relevant to the national heritage of the Republic of Uzbekistan.

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