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Phylogenetic analysis of a region of mitochondrial *cox-1* as a DNA barcode marker sequence for the Siberian ibex *Capra sibirica* (Bovidae: Mammalia) in Mongolia

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Abstract

Siberian ibex or Altain yangir, *Capra sibirica* Pallas from Central Asia is believed to be the most ancient species of the genus *Capra*. In Mongolia, it is distributed in the areas of the Mongolian Altai, Gobi-Altai, Dzungaria, Altai, Khan Khuhii, Khoridal Saridag, and Ulaan Taiga as well as in the desert and semi-desert steppe zones of Dundgobi and Dornogobi provinces. *Capra sibirica*, a near-threatened species, is affected by illegal hunting for meat and sport. The mitochondrial *cytochrome c oxidase subunit 1* gene (*cox-1*) is used as a DNA marker to distinguish mammalian species for the investigation of illegal hunting. In this study, we sequenced a part of the *cox-1* of eight Mongolian *Capra sibirica* individuals. Our DNA sequences were clustered in a clade of *Capra* which is distinct from other clades of mammalian species in the phylogenetic tree. Our findings suggest that the DNA sequences can be utilized for the investigation of illegal hunting.

Key words: Altain yangir, *Capra*, DNA barcoding system, illegal hunting, near threatened species

The Siberian ibex, *Capra sibirica* Pallas is a near-threatened species belonging to the genus *Capra*, subfamily Caprinae, and family Bovidae (Reading et al., 2020). *Capra sibirica* can be found in portions of Mongolia, Afghanistan, China, India, Kazakhstan, Kyrgyzstan, Pakistan, Russia, Tajikistan, and Uzbekistan. Across its range, it is primarily found in mountainous and rocky terrain (Fedosenko and Blank, 2001). In Mongolia (MGL), they are found in much of the Altai and Gobi-Altai Mountain ranges, in parts of the Khangai Mountains, in some ranges in northwestern Mongolia (the red taiga mountains of Khoridal Saridag in Khuvsgul aimag), and in mountains in the Gobi and Tov Province (Mallon, 1985). In the early 1980s, the Siberian ibex population was estimated at 80,000 in the "The Mongolian Red Data Book" in 1987 (Shagdarsuren et al., 1987). A 2009 national estimation report of Mongolian mountain ungulates showed that the Siberian ibex was distributed over a total area of 55,985 km^2 and the population was estimated as 36,000 in the country (Lkhagvasuren, 2010). According to the results of the above survey, the *Capra sibirica* population has dramatically declined

in the last years due to illegal hunting, natural predators, competition with livestock for resources, infectious diseases, habitat degradation (coal mining), and global warming. However, the current total number of individuals in Mongolia is unclear due to the difficulty of counting free-roaming wild animals. Illegal hunting for meat and sport remains out of control due to a lack of appropriate investigation methods. To investigate illegal hunting, the DNA Barcoding System (DBS) aids in identifying animal species using a biological specimen obtained from meat, blood, hair, bone, or other biological samples. The mitochondrial *cytochrome c oxidase I* gene (*cox-1*) is one of the suitable genetic markers widely used in DBS (Rodrigues et al., 2017).

In the present study, we aim to develop a DNA barcoding system using different types of biological samples to investigate illegal hunting of *Capra sibirica* based on the sequence of the *cox-1* gene.

Sampling locations and DNA extraction

All experiments were approved by the Ethical Committee of the National University of Mongolia (approval number: ШУ/УЗ-2020/02). Tissue samples of eight individuals of *Capra sibirica* were collected from 5 provinces in Mongolia (Fig. 1 and Table 1 in the supplementary material). The map (Fig. 1) was generated using ArcGIS 10.4 version (California, United States). Twenty-five-gram tissue samples were collected from animals that died due to snow and extreme cold. The study was done between 2020 and 2024. All samples were kept at -80 °C in the National Institute of Forensic Sciences as fresh tissue samples until used for DNA sequencing. A 5-gram tissue sample was ground in liquid nitrogen and lysed by cell lysis buffer (20 mM Tris-Cl pH 8.0, 5 mM EDTA pH 8.0, 400 mM NaCl, 1% SDS) with proteinase K at 55 ℃ for 2 hours. Protein was removed by equilibrated phenol and chloroform: isoamyl alcohol (24:1). The supernatant containing DNA was then precipitated using 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate. The resulting genomic DNA pellet was dissolved in DNase-free grade water (Bayarlkhagva et al., 2023).

DNA amplification and sequencing

A polymerase chain reaction (PCR) mixture was prepared, consisting of 1 × Takara PCR buffer, 200 µM dNTPs, 1 µM of forward primer (VF1d: 5′-TCTCAACCAACCACAARGAYATYGG-3′) and reverse primer (VR1d: 5′-TAGACTTCTGGGTGGCCRAARAAYCA-3′) as previously designed (Ivanova et al., 2006), 0.2U Takara DNA polymerase (Kusatsu, Japan), nuclease-free grade water, and the genomic DNA. PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 1 min and the final extension at 72 °C for 10 min (Ivanova et al., 2006). PCR amplicons (expected size of amplicon is 709 bp) were separated in 1.2% agarose gel stained with 1% ethidium bromide. The amplicons were then cut and extracted using the NucleoSpin Gel and PCR Clean-up, Mini kit (Duren, Germany), following the manufacturer's instructions. The nucleotide sequences of *cox-1* were sequenced using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit (California, USA), according to the manufacturer's instructions. The amplicons were then sequenced, and the sequencing reads generated by the forward and reverse primers were assembled and trimmed based on the quality of each nucleotide, as determined from the chromatographs (Ivanova et al., 2006). The obtained sequences were deposited in GenBank for further use as a DNA barcode marker sequence.

Phylogenetic analysis

The phylogenetic tree of *C. sibirica cox-1* was constructed using Molecular Evolutionary Genetics Analysis version 10.0 (MEGA X) (Kumar et al., 2018). Additional nucleotide sequences retrieved from GenBank were included in the phylogenetic analysis, and the accession numbers are listed in Table 2 in the supplementary material. The exact aligned matrix length was 535 bp. Among the 48 sequences analyzed, we used 18 sequences of *Capra sibirica*, two sequences of *Capra hircus* (Linnaeus), three sequences of *Capra aegagrus* (Erxleben), three sequences of *Capra falconeri* (Wagner), two sequences of *Hemitragus jemlahicus* (Smith), two sequences of *Ovis aries* (Linnaeus), three sequences of *Pantholops hodgsonii* (Abel), two sequences of *Gazella subgutturosa* (Güldenstädt), four sequences of *Saiga tatarica* (Linnaeus), two sequences of *Procapra picticaudata* (Hodgson), two sequences of *Procapra prezewalskii* (Büchner), and four sequences of *Procapra gutturosa* (Pallas).

The best model, the Hasegawa-Kishino-Yano (HKY) model for the phylogenetic tree, was determined using the maximum likelihood statistical method (Hasegawa et al., 1985). For the outgroup, a sequence of *Camelus bactrianus* Linnaeus (accession number: MZ049301) was provided. A bootstrap value of 1,000 was set to test the variants of the phylogenetic tree (Felsenstein, 1985).

Capra is a genus of mammals, the goats, composed of up to nine species, including *Capra sibirica* and many species known as ibexes. The domestic goat (*Capra hircus*) is a domesticated species derived from the wild goat (*Capra aegagrus*) (Pidancier et al., 2006).

In this study, we successfully amplified a specific amplicon of *cox-1* with the expected size of 709 base pairs (bp), from 8 DNA samples of *Capra sibirica*. After error correction, the sequence lengths ranged from 535 to 628 bp, as listed in supplementary Table 1.

Table 1: The sampling location, coordinates and accession numbers of Mongolian *Capra sibirica* cox-1 gene.

Province	Coordinates	Isolate	GenBank accession number	DNA barcode sequence size (bp)
Khovd, Bulgan	N 46.057584 E 91.592069	MGL-05	KU527896	602
Bayan-Ulgii, Buyant	N 48.5791667 E 89.55	MGL-33	OP967516	625
Gobi-Altai, Erdene	N 45.0869306 E 97.37000277777777	MGL-67	OP967905	562
Bayankhongor Shinejinst	N 44.5468695 E 99.2796975	MGL-238	OP967918	627
Bayankhongor, Khureemaral	N 46.460875 E 98.2613184	MGL-239	OP236735	562
Khovd, Sutai	N 46.52597606367025 E 93.6197283968014	MGL-240	OP236739	628
Bayan-Ulgii, Altai Tavan bogd	N 48.42367357138719 E 89.54154089829662	MGL-244	OP967922	573
Umnugobi, Gurvansaikhan	N 43.656918057557256 E 103.55696298602012	MGL-401	OR539442	535

Table 2: Sequences of mitochondrial cytochrome c oxidase subunit 1 gene (cox-1) retrieved from Genbank used to construct the phylogenetic tree.

Region	Country	GenBank accession number	Isolate
Asia	Mongolia	OP967922.1 *Capra sibirica*	MGL-244
	Mongolia	OR539442 *Capra sibirica*	MGL-401
	Mongolia	MW546057.1 *Capra sibirica*	CO42 (cytochrome c oxidase sample no.42) (Hacker et al., 2021 unpublished)
	Mongolia	OP967905.1 *Capra sibirica*	MGL-67
	Mongolia	OP967918.1 *Capra sibirica*	MGL-238
	Mongolia	OP236739.1 *Capra sibirica*	MGL-240
	Mongolia	AB743816.1 *Capra sibirica*	G1253 (Nomura et al., 2013)
	Mongolia	OP967516.1 *Capra sibirica*	MGL-33
	Mongolia	KU527896.1 *Capra sibirica*	MGL-05
	Mongolia	OP236735.1 *Capra sibirica*	MGL-239
	Pakistan	OK244602.1 *Capra sibirica*	UNGPK041 (Naseem, 2022, unpublished)
	Pakistan	MT251399.1 *Capra sibirica*	HI-BRC3 (Naseem, 2020, unpublished)
	Pakistan	MT251398.1 *Capra sibirica*	HI-BRC2 (Naseem, 2020, unpublished)
	Pakistan	MH261363.1 *Capra sibirica*	PBRC-SibIB M4f (Naseem et al., 2019, unpublished)
	Pakistan	MT251400.1 *Capra sibirica*	HI-BRC4 (Naseem, 2020, unpublished)
	Pakistan	OK244603.1 *Capra sibirica*	UNGPK042 (Naseem, 2022, unpublished)
	Pakistan	OK244601.1 *Capra sibirica*	UNGPK040 (Naseem, 2022, unpublished)
	Pakistan	OK244600.1 *Capra sibirica*	UNGPK039 (Naseem, 2022, unpublished)
	Pakistan	MT251384.1 *Capra aegagrus*	SI-J3 (Naseem, 2020, unpublished)
	Pakistan	MT251383.1 *Capra aegagrus*	SI-J2 (Naseem, 2020, unpublished)
	Pakistan	MT251386.1 *Capra aegagrus*	SI-J5 (Naseem, 2020, unpublished)
	Pakistan	MT251388.1 *Capra falconeri*	AMK-GB2 (Naseem, 2020, unpublished)
	Pakistan	OK244589.1 *Capra falconeri falconeri*	UNGPK028-19 (Naseem, 2022, unpublished)
	Pakistan	MG742698.1 *Capra falconeri falconeri*	PBRC-MK M4f106 (Naseem et al., 2018, unpublished)
	New Zealand	KF317915.1 *Hemitragus jemlahicus*	NT6T (Ramón-Laca et al., 2014)
	New Zealand	KF317904.1 *Hemitragus jemlahicus*	MT5T (Ramón-Laca et al., 2014)
	Mongolia	OM540944.1 *Gazella subgutturosa*	MGL-70
	Mongolia	KX882052.1 *Gazella subgutturosa*	MGL-90 (2018)
	China	KC679008.1 *Procapra picticaudata*	TB1 (Chen et al., 2015)
	China	KC679009.1 *Procapra picticaudata*	TB2 (Chen et al., 2015)
	China	KC679051.1 *Procapra przewalskii*	SG6 (Chen et al., 2015)
	China	KC679050.1 *Procapra przewalskii*	SG1 (Chen et al., 2015)
	Mongolia	KU922905.1 *Procapra gutturosa*	MGL-23 (2016)
	Mongolia	KU527899.1 *Procapra gutturosa*	MGL-08 (2016)
	Mongolia	KU527898.1 *Procapra gutturosa*	MGL-07 (2016)
	Mongolia	KU527895.1 *Procapra gutturosa*	MGL-04 (2016)
	Mongolia	KX859292.1 *Saiga tatarica*	MGL-162 (2016)
	China	KC679013.1 *Saiga tatarica*	Saiga4 (Chen et al., 2015)
	China	KF735210.1 *Saiga tatarica*	LYBZ01YP (Zhang et al., 2015, unpublished)
	China	KF735178.1 *Saiga tatarica*	LYZJ01AA (Zhang et al., 2015, unpublished)
	China	HQ269461.1 *Pantholops hodgsonii*	m305 (Cai et al., 2011)
	China	KJ862178.1 *Pantholops hodgsonii*	A53 (Li et al., 2015, unpublished)
	China	HQ269460.1 *Pantholops hodgsonii*	m304 (Cai et al., 2011)
	Mongolia	KX859288.1 *Ovis aries*	MGL-152 (Bayarlkhagva et al., 2018, unpublished)
	China	HQ269453.1 *Capra hircus*	shyjd2 (Cai et al., 2011)
	China	MZ049301.1 *Camelus bactrianus*	Bactrian_camel8-F-*coi*-1 (Zhang et al., 2022, unpublished)
Africa	Egypt	MG571555.1 *Capra hircus*	Breed Dimashki (El-Gendy et al., 2017, unpublished)
North America	USA	JN850771.1 *Ovis aries*	Sheep_1 (Thiemann et al., 2012)

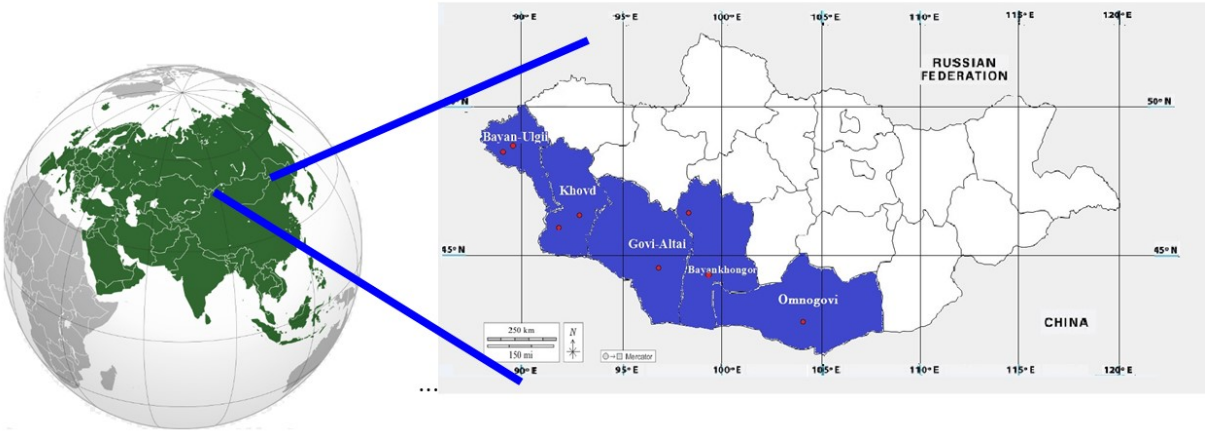

Figure 1: The sampling locations of eight *Capra sibirica* individuals from five provinces (Bayan-Ulgii, Khovd, Gobi-Altai, Omnogovi, and Bayankhongor) in Mongolia, Central Asia. One specimen was collected from the Gobi-Altai (MGL67), and Omnogovi Province (MGL401), while two specimens from each province were obtained from the Bayan-Ulgii (MGL33, MGL244), Khovd (MGL05, MGL240), and Bayankhongor (MGL238, MGL239) provinces. The red dots on the map indicate the sampling locations. [Source: The map was generated using ArcGIS 10.4 version (California, United States)].

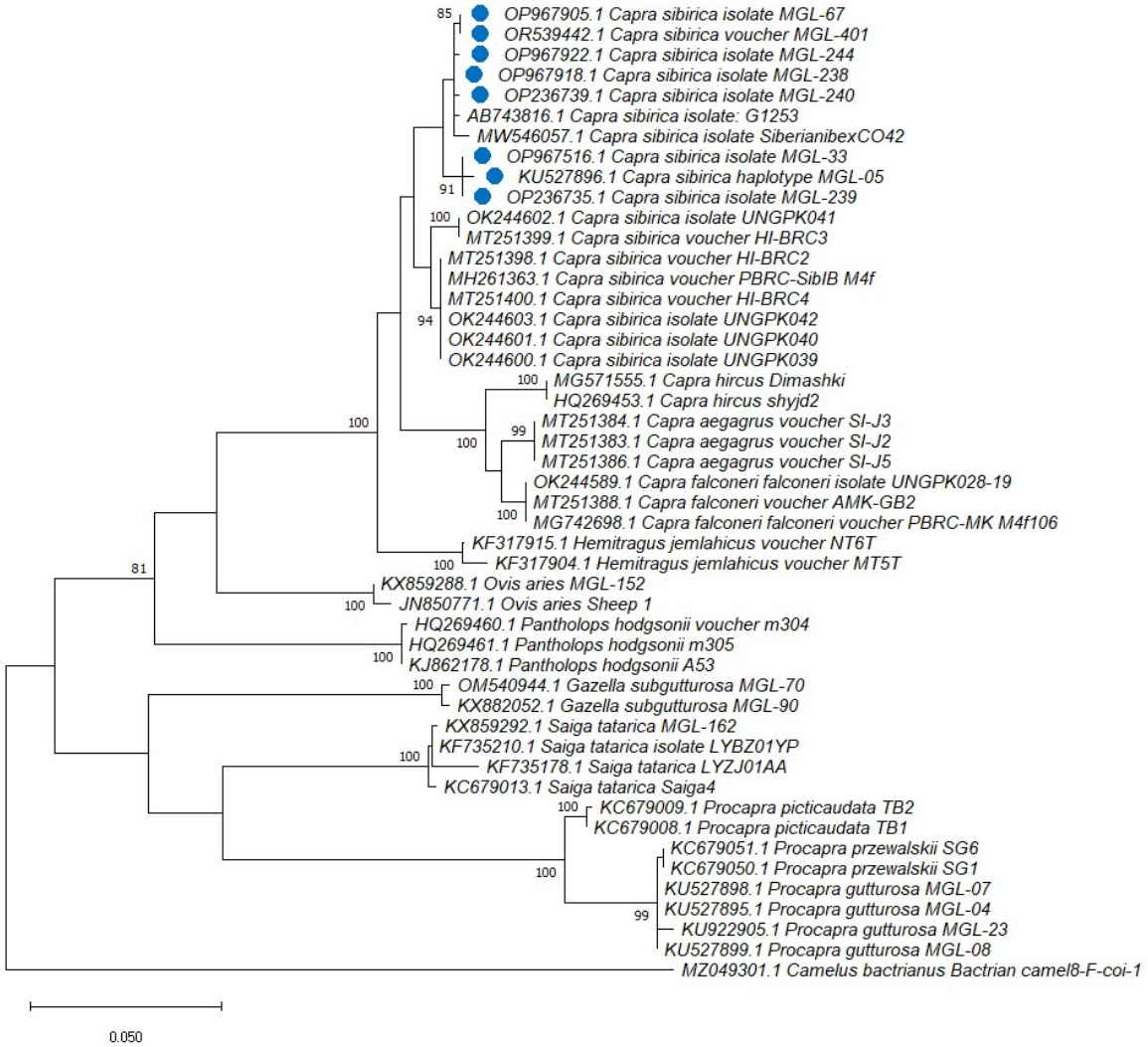

Figure 2: The phylogenetic tree was constructed using partial sequences of *cox-1* of the mitochondrial genomic DNA of *Capra sibirica* and other mammalian species. The tree was constructed using the best model, the Hasegawa-Kishino-Yano model. A bootstrap value of 1,000 replications was used for statistical analysis. Only values higher than 75% are shown on the phylogenetic tree.

In the phylogenetic tree, a total of 48 sequences, including Mongolian *C. sibirica*, were assembled into their respective clades (Fig. 2). The *cox-1* sequence of *C. bactrianus* was established as the outgroup, as expected. Only 18 *cox-1* sequences of *C. sibirica* isolates are available in GenBank, including our 8 sequences from Mongolian *Capra sibirica* isolates. We have added a significant number of sequences, from a different region, to what is available for the species. Among the remaining 10 isolates, 8 were registered from Pakistan, and 2 isolates were from Mongolia (Hacker et al., 2021 unpublished data, (Nomura et al., 2013)). Regarding the Mongolian isolates, all 10 isolates (MGL-05, MGL-33, MGL-67, MGL-238, MGL-239, MGL-240, MGL-244, MGL-401, G1253 (Nomura et al., 2013), and CO42 (cytochrome c oxidase sample no.42) including our 8 isolates from 5 provinces, cluster as two subclades. Exact locations of G1253, and CO42 are unknown. Three isolates (MGL-05, MGL-33, and MGL-239) were genetically closer to each other (Fig. 2). The remaining 8 Pakistan isolates clustered together, forming another subclade within the *Capra* clade. Mongolia and Pakistan are geographically distant, separated by China and unfortunately, there is no registered *cox-1* data from China so far.

Based on our phylogenetic analysis of the *cox-1*, *Capra sibirica* can be distinguished from other wild and domestic animal species, particularly from livestock such as *O. aries* (sheep), *C. hircus* (goats), and, obviously, from *C. bactrianus* (camels). This indicates that the sequenced region of *cox-1* can be used as a useful DNA marker sequence to enrich the dataset of DBS for identifying illegally hunted meat products of *Capra sibirica* among mutton and other livestock-derived meat products.

Interestingly, the three Mongolian isolates (MGL-33, MGL-05 and MGL-239) from 3 different provinces (Bayan-Ulgii, Khovd, and Bayankhongor, respectively) cluster as a subclade within the *Capra* clade. Their close genetic relationship and distant location suggest that they belong to a specific population that lives in a large territory. This population might be isolated from other Mongolian populations of *C. sibirica* due to a geographic barrier. This geographic barrier may impede gene flow and probably increases inbreeding within the isolated population (Chen et al., 2023). Future studies are needed to investigate gene flow events and potential inbreeding in this particular population of *C. sibirica*.

In conclusion, we identified Mongolian *C. sibirica* isolates within the *Capra* clade based on the analysis of the mitochondrial *cytochrome c oxidase subunit 1* gene. The sequence of *cytochrome c oxidase subunit 1* gene is a suitable marker for a DNA Barcoding System, enabling the differentiation between illegally hunted *C. sibirica* specimens and livestock-derived specimens.

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Author contributions

Bayarlkhagva Damdingiin was the project leader. Munkhjargal Bayarlkhagva, Bolortuya Ulziibat, Bayarmaa Gun-Aajav isolated DNA samples and did the PCR and sequencing. Munkhjargal Bayarlkhagva developed the results, registered sequences to NCBI and prepared a press summary of the project. Davaa Bazarsad prepared samples. Enkhbaatar Batmagnai constructed the phylogenetic tree and wrote the manuscript of the article for publication, including tables and figures.

Conflicts of interest

The authors declare that there are no conflicting issues related to this short communication.

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