museu de ciències naturals de Barcelona

Animal Biodiversity and Conservation 46.2 (2023)
DOI: 10.32800/abc.2023.46.0213

Research paper

Phylogenetic analysis of a region of mitochondrial cox-1 as a DNA barcode marker sequence of *Gazella subgutturosa* (goitered gazelle) in Mongolia

M. Bayarlkhagva[1], B. Ulziibat[2], B. Gun-Aajav[3], D. Bazarsad[4], B. Damdingiin[3], E. Batmagnai[5]

Author affiliations:
[1] Zool Soc of London's Representative Office in Mongolia
[2] Inst of Biology, Mongolian Academy of Sciences
[3] Nat Univ of Mongolia
[4] Nat Inst of Forensic Science
[5] Inst of Veterinary Medicine, Mongolian Univ of Life Sciences

Corresponding author:
Enkhbaatar Batmagnai
magnaishuudan@gmail.com

Handling Editor:
Susana González

Received: 25/05/2023
Cond. acceptance: 16/10/2023
Final acceptance: 23/10/2023
Published: 03/11/2023

Cite:
Bayarlkhagva M, Ulziibat B, Gun-Aajav B, Bazarsad D, Damdingiin B, Batmagnai E, 2023. Phylogenetic analysis of a region of mitochondrial *cox-1* as a DNA barcode marker sequence of *Gazella subgutturosa* (goitered gazelle) in Mongolia. *Animal Biodiversity and Conservation* 46.2, 213-217. DOI: http://doi.org/10.32800/abc.2023.46.0213

ISSN: 1578-665 X
eISSN: 2014-928 X

Abstract

Phylogenetic analysis of a region of mitochondrial cox-1 *as a DNA barcode marker sequence of* Gazella subgutturosa *(goitered gazelle) in Mongolia. Gazella subgutturosa*, a vulnerable species, is threatened 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* gene (709 bp) of six Mongolian *G. subgutturosa* individuals. Our DNA sequences were clustered in a clade of *Gazella* which is distinct from other clades of mammalian species in the phylogenetic tree. Our findings suggest that DNA sequences can be useful in the investigation of illegal hunting.

Key words: Vulnerable species, *Gazella subgutturosa*, *cox-1*, Phylogenetic analysis, DNA Barcoding System, Mongolia

Resumen

Análisis filogenético de una región del gen mitocondrial cox-1, *una secuencia marcadora del código de barras de* Gazella subgutturosa *(gacela persa) en Mongolia*. *Gazella subgutturosa* es una especie vulnerable afectada por la caza ilegal para la obtención de carne y como actividad deportiva. El gen mitocondrial de la subunidad 1 de la citocromo c oxidasa (*cox-1*) se utiliza como marcador de ADN para distinguir especies de mamíferos en el ámbito de la investigación de la caza ilegal. En este estudio, secuenciamos una parte del gen *cox-1* (709 pb) de seis ejemplares de gacela persa, *Gazella subgutturosa*. Nuestras secuencias de ADN se agruparon en un clado de *Gazella* que es diferente de otros clados de especies de mamíferos del árbol filogenético. Nuestros resultados sugieren que las secuencias de ADN se pueden utilizar para la investigación de la caza ilegal.

Palabras clave: Especie vulnerable, *Gazella subgutturosa*, *cox-1*, Análisis filogenético, Sistema de código de barras del ADN, Mongolia

Introduction

Gazella subgutturosa is a vulnerable species belonging to the genus *Gazella*, subfamily Antilopinae, and family Bovidae (IUCN SSC Antelope Specialist Group, *Gazella subgutturosa* 2017; Munkhbat 2016). The distribution of *G. subgutturosa* is limited to Asia and the Middle East, including Mongolia, Turkey, the Syrian Arab Republic, Saudi Arabia, Bahrain, Jordan, Iraq, Yemen, the Islamic Republic of Iran, Azerbaijan, Kazakhstan, United Arab Emirates, Oman, Turkmenistan, Uzbekistan, Afghanistan, Pakistan, Tajikistan, Kyrgyzstan, and China (IUCN SSC Antelope Specialist Group, *Gazella subgutturosa*, 2017). The population of *G. subgutturosa* has undergone gradual decline over the last 50 years due to illegal hunting, natural predators, competition with livestock for resources, infectious diseases, habitat degradation (coal mining), and global warming (Lkhagvasuren et al 2001).

Approximately 40-50% of the global population of *G.subgutturosa* graze in Mongolia (Lkhagvasuren et al 2001). The distribution of *G. subgutturosa* has been reported in a total area of 343,982 square km in 52 districts in the southern provinces of Mongolia (Oyun and Shiirevdamba 2014). Between the 1940s and 1960s, the population of this species decreased by 30% (Lkhagvasuren et al 2001). In 1990, the population size was estimated at 60,000 individuals (Amgalan 1995). A more recent study, in 2013, estimated a total of 28,462 (95% CI 21,326-37,987) individuals in parts of Omnogovi and Dornogovi provinces (Buuveibaatar et al 2017). However, the current total number of individuals in Mongolia is unclear due to the difficulty in counting free-roaming wild animals. Although all hunting of *G. subgutturosa* is prohibited by the Mongolian government Act No. 264 (2001) (Clark and Munkhbat 2006), illegal hunting for meat and sport remains out of control for reasons that include a lack of appropriate investigation methods that would provide more evidence about hunted species. To investigate illegal hunting, the DNA Barcoding System (DBS) helps to identify animal species using a biological specimen obtained from meat, blood, hair, bone, or other biological samples. DBS could be used in court proceedings for illegal hunts. The mitochondrial cytochrome c oxidase I gene (*cox-1*) is one of the suitable genetic markers widely used in DBS. *G. subgutturosa* should be differentiated from *Procapra gutturosa*, *Procapra* picticaudata, *Procapra prezewalskii*, and *Saiga tatarica*. When we distinguish meat or small portions of biological samples we should distinguish between camel, sheep, goat, horse and cattle because Mongolia has around 70 million livestock including these domestic animals.

In the present study, we sequenced a part of *cox-1* from six individuals of *G. subgutturosa*. The obtained sequences were deposited in GenBank for further use as a DNA barcode marker sequence.

Material and methods

Sampling locations and DNA extraction

All experiments were approved by the Ethical Committee of the National University of Mongolia. Three specimens (MGL70, MGL163, MGL164) were collected from the Govi-Altai province, while the remaining three specimens (MGL06, MGL90, MGL91) were obtained from the Dornogovi province (fig. 1 and table 1s in the supplementary material). Twenty-five–gram tissues samples were collected from animals that died due to snow and extreme cold. A 5-gram of 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 °C 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 3 M acetate Na. The resulting genomic DNA pellet was dissolved in DNase-free grade water.

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), 0.2 U 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. PCR amplicons (expected size of amplicon is 709 bp) were separated in 1.2% agarose gel stained with 1% of 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.

Phylogenetic analysis

The phylogenetic tree of *G. subgutturosa 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, origins of the sequences are listed in table 2s in the supplementary material. The exact aligned matrix length was 512 bp. Among the 42 sequences analyzed, we used 17 sequences of *G. subgutturosa*, five sequences of *Procapra gutturosa*, four sequences of *Procapra* picticaudata, three sequences of *Procapra prezewalskii*, three sequences of *Saiga tatarica*, three sequences of *Pantholops hodgsonii*, two sequences of *Ovis aries*, and two sequences of *Capra hircus*. *G. subgutturosa* should be differentiated from *Procapra gutturosa*, *Procapra* picticaudata, *Procapra prezewalskii*, *Saiga tatarica*, and *Pantholops hodgsonii*.

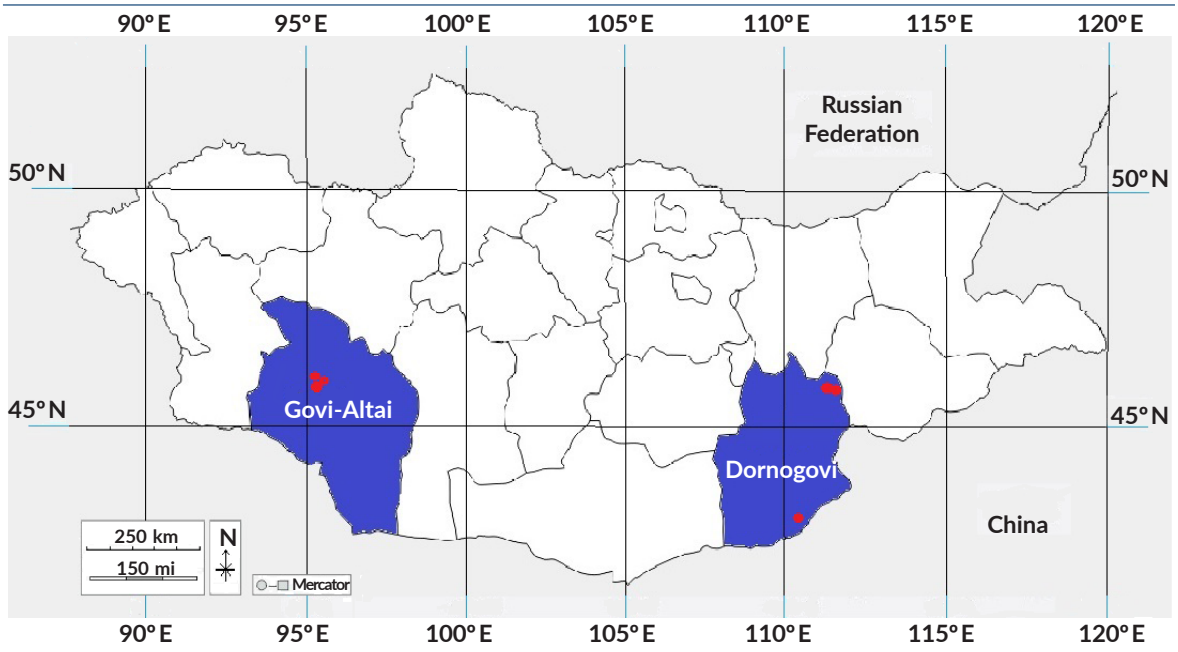

Fig. 1. The sampling locations of six *Gazella subgutturosa* individuals from five locations in Mongolia. The red dots on the map indicate the sampling locations.

Fig. 1. *Sitios de muestreo de seis ejemplares de* Gazella subgutturosa *en cinco lugares diferentes de Mongolia. Los puntos rojos del mapa indican los sitios de muestreo.*

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, two sequences of *Camelus bactrianus* (accession number: MZ049301) and *Equus caballus* (accession number HM102300) were provided. A bootstrap value of 1,000 was set to test the variants of the phylogenetic tree (Felsenstein 1985).

Results and discussion

The global population of *G. subgutturosa*, a vulnerable species, has gradually halved in Mongolia over the past 50 years (Lkhagvasuren et al 2001). Research into illegal hunting contributes significantly to protecting vulnerable mammalian species. DBS is one of the advanced approaches established based on DNA marker sequences. In this study, we successfully amplified a specific amplicon of *cox-1* with the expected size of 709 base pairs (bp), from all DNA samples of *G. subgutturosa*. After error correction, the sequence lengths ranged from 466 to 664 bp, as listed in table 1s in the supplementary material.

In the phylogenetic tree, a total of 42 sequences, including Mongolian *G. subgutturosa*, were classified into their respective clades (fig. 2). Additionally, a sequence of *Bos taurus* was classified as a sister clade of *Capra hircus*, *Ovis aries* and *Pantholops hodgsonii*, consistent with the classification observed in a previous study (Chen et al 2015). The *cox-1* sequences of *Equus caballus* and *Camelus bactrianus* were classified as outgroups, as expected.

Only 17 *cox-1* sequences of *G. subgutturosa* isolates are available in the GenBank, including our 6 sequences from Mongolian *G. subgutturosa* isolates. We have added a significant number of sequences, from a different region, to what is available for the species. Among the remaining 11 isolates, two were recorded from Pakistan, and nine from China. Regarding the Mongolian isolates, three out of six isolates (MGL-70, MGL-163, and MGL-164) from Govi-Altai province clustered together as a subclade within the *Gazella* clade. The remaining three Mongolian isolates (MGL-06, MGL-90, and MGL-91) from Dornogovi province, clustered together with Chinese isolates (Ka3, Ka5, and Ka9) as previously recorded (Chen et al 2015). However, the relationships between the Mongolian isolates (MGL-06, MGL-90, and MGL-91) and the Chinese isolates (Ka3, Ka5, and Ka9) are difficult to explain for the time being because the exact locations of Ka3, Ka5, and Ka9 isolates are unclear and statistical support to reveal phylogenetic relationships among them is lacking.

Based on our phylogenetic analysis of the *cox-1*, the *G. subgutturosa* species can be distinguished from other wild and domestic animal species, particularly from livestock such as *B. taurus* (cattle), *O. aries* (sheep), *C. hircus* (goats), *C. bacterianus* (camels) and *E. caballus* (horse). This indicates that the sequenced region of *cox-1* can be a useful DNA marker sequence to enrich the dataset of DBS to identify illegally hunted meat products of *G. subgutturosa* among beef and other livestock-derived meat products.

Apart from other Mongolian specimens, the three Mongolian isolates from Govi-Altai province clustered

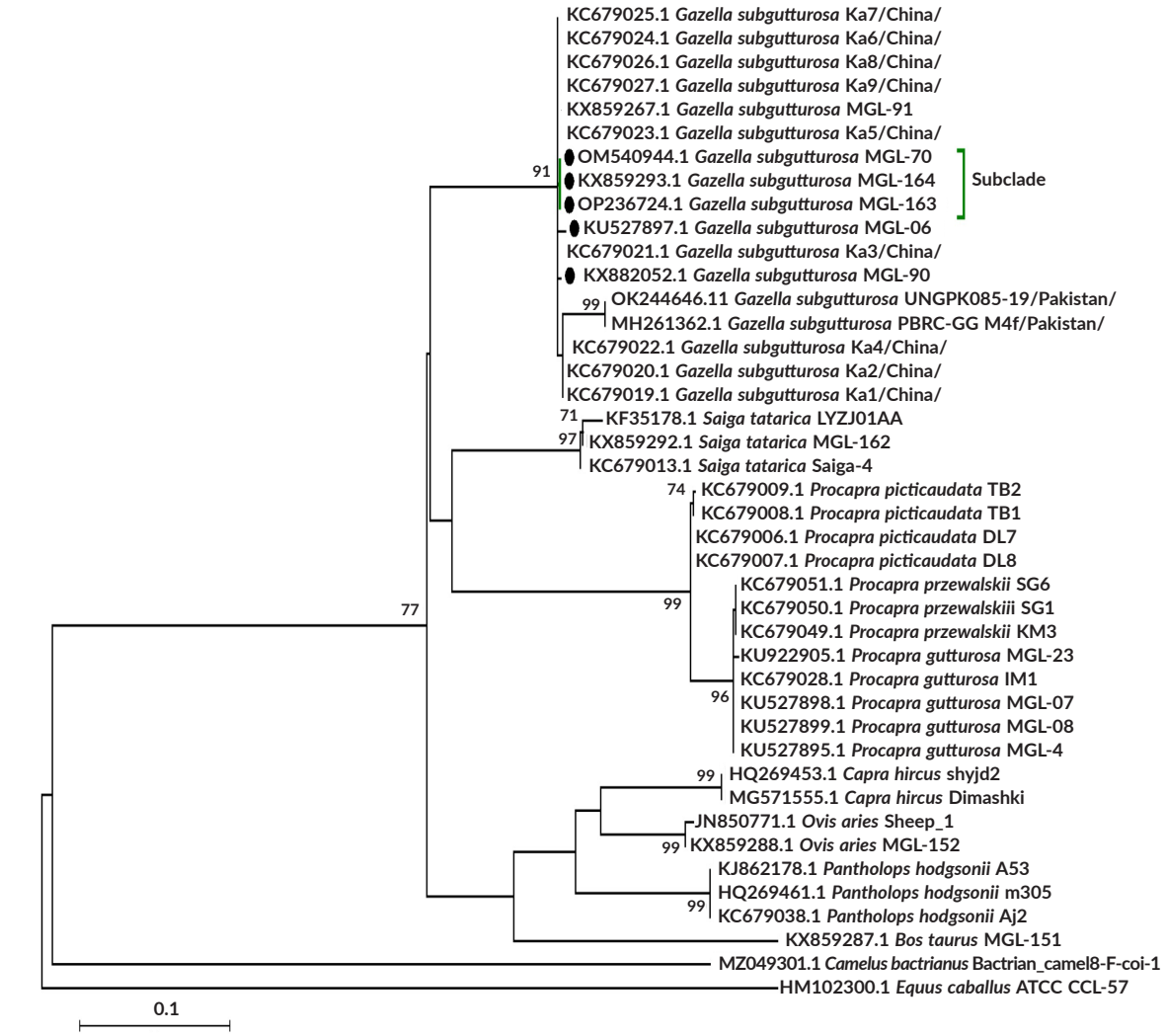

Fig. 2. The phylogenetic tree was constructed using partial sequences of cox-1 of the mitochondrial genomic DNA of *Gazella subgutturosa* and other mammalian species. The tree was constructed using the best model, the Hasegawa-Kishino-Yano model. The black dots in the phylogenetic tree indicate Mongolian *G. subgutturosa* isolates. A bootstrap value of 1,000 replications was used for statistical analysis. Values lower than 70 % were hidden in the phylogenetic tree.

Fig. 2. *El árbol filogenético se elaboró utilizando secuencias parciales del ADN genómico mitocondrial del gen cox-1 de* Gazella subgutturosa *y otras especies de mamíferos. Para elaborarlo se recurrió al mejor modelo, el de Hasegawa-Kishino-Yano. Los puntos negros del árbol filogenético indican los grupos aislados de* G. subgutturosa. *Para realizar el análisis estadístico, se utilizó un valor de bootstrap de 1.000 repeticiones. Los valores inferiores al 70 % no se muestran en el árbol filogenético.*

as a subclade within the *Gazella* clade. Their close genetic relationship and shared location suggest that they belong to a specific population. This population might be isolated from other Mongolian populations of *G. subgutturosa* because to Govi-Altai province is located in the high mountainous region in the north-eastern part of the Altai Mountain. This geographic barrier may impede gene flow and promote inbreeding within the isolated population. Future studies are needed to investigate gene flow events and potential inbreeding in this particular population of *G. subgutturosa*.

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

References

Amgalan L, 1995. *Distribution and population dynamics of goitered gazelle* (Gazella subgutturosa) *in Mongolia*. Report from the conference 'Biology-30 years'. Institute of Biology of the Mongolian Academy of Sciences, Ulaanbaatar. (In Mongolian).

Buuveibaatar B, Strindberg S, Kaczensky P, Payne J, Chimeddorj B, Naranbaatar G, Amarsaikhan S, Dashnyam B, Munkhzul T, Purevsuren T, Hosack DA, Fuller TK, 2017. Mongolian Gobi supports the world's largest populations of khulan *Equus hemionus* and goitered gazelles *Gazella subgutturosa*. *Oryx* 51, 639-647. DOI: 10.1017/S0030605316000417

Chen J, Jiang Z, Li C, Ping X, Cui S, Tang S, Chu H, Liu B, 2015. Identification of ungulates used in a traditional Chinese medicine with DNA barcoding technology. *Ecology and Evolution* 5, 1818-1825. DOI: 10.1002/ece3.1457

Clark EL, Munkhbat J, 2006. *Summary Conservation Action Plans for Mongolian Mammals Compiled by Emma L*. Clark and Munkhbat Javzansuren.

Felsenstein J, 1985. *Confidence Limits on Phylogenies: An Approach Using the Bootstrap*. Evolution (NY) 39, 783. DOI: 10.2307/2408678

Hasegawa M, Kishino H, Yano, T-aki, 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* 22, 160-174. DOI: 10.1007/BF02101694

IUCN SSC Antelope Specialist Group, *Gazella subgutturosa*, 2017. *IUCN Red List Threat*. *Species*. DOI: 10.2305/IUCN.UK.2017-2.RLTS.T8976A50187422.en

Ivanova NV, Dewaard JR, Hebert PDN, 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes* 6, 998-1002. DOI: 10.1111/j.1471-8286.2006.01428.x

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, 1547-1549. DOI: 10.1093/molbev/msy096

Lkhagvasuren B, Dulamtseren S, Amgalan L, 2001. Mongolia. In: *Antelopes. Part 4: North Africa, the Middle East and Asia*: 159-167 (DP Mallon, SC Kingswood Eds). Global Survey and Regional Action Plans. IUCN, Gland and Cambridge, IUCN/SSC Antelope Specialist Group.

Munkhbat J, 2016. *Mongolian Red List of Mammals Mongolian Red List of Mammals Compiled by Emma L* . Clark and Munkhbat, Javzansuren.

Oyun S, Shiirevdamba T, 2014. *Mongolian Red book*. Ministry of Environment and Green Development.

Acknowledgements
The study is a part of the project titled "Introducing mitochondrial DNA technology into the practice of forensic analysis against illegal hunting and animal theft" which was commissioned by the Ministry of Justice and Internal Affairs. Funders did not have any role in the present study or in the preparation of the manuscript. The authors express their gratitude to Dr. Bumduuren Tuvshintulga (PhD) for providing valuable guidance in writing this article

Author contributions
M Bayarlkhagva, B Ulziibat, B Gun-Aajav isolated DNA samples and done a PCR and sequencing. **M Bayarlkhagva** developed the results, registered sequences to NCBI and prepared a press summary of the project. **D Bazarsad** prepared samples. **B Damdingiin** was the project leader. **E Batmagnai** prepared the article for publication, including figures, phylogenetic analysis

Conflicts of interest
No conflicts declared

Funding
This work was supported by the Science and Technology Foundation of Mongolia (approval number: ШУУ/У3-2020/02)

Complete affiliations
M Bayarlkhagva, Zoological Society of London's Representative Office in Mongolia, Ulaanbaatar, Mongolia
B Ulziibat, Plant biotechnology laboratory, Institute of Biology, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia
B Gun-Aajav, B Damdingiin, Department of Biology, School of Arts and Sciences, National University of Mongolia, Ulaanbaatar, Mongolia
D Bazarsad, Department of Scientific Analysis, National Institute of Forensic Science, Ulaanbaatar, Mongolia
E Batmagnai, Institute of Veterinary Medicine, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia

Supplementary material

Table 1s. The sampling location, coordinates, and accession numbers of Mongolian *G. subgutturosa cox-1* gene.

Tabla 1s. *Sitio de muestreo, coordenadas y número de accesión del gen* cox-1 *de* G. subgutturosa.

Province	Coordinates	Isolate	GenBank accession number	DNA barcode sequence size (bp)
Dornogovi	N 43° 26' 30.89'' E 110° 42′ 8.69''	MGL-06	KU527897	605
Dornogovi	N 45°81' 61.03' E 111° 81' 73.83''	MGL-90	KX882052	466
Dornogovi	N 45° 81' 61.03'' E 111° 81' 73.83''	MGL-91	KX859267	655
Govi-Altai Khuisiin govi	N 46° 15' 9.31'' E 95° 15' 33.49''	MGL-70	OM540944	664
Govi-Altai Tugrug district	N 46° 09' 0.31'' E 95° 25' 4.61''	MGL-163	OP236724	616
Govi-Altai Shargiin govi	N 46° 01' 2.88'' E 95° 48' 5.86''	MGL-164	KX859293	664

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

Tabla 2s. *Secuencias del gen mitocondrial de la subunidad 1 de la citocromo c oxidasa* (cox-1) *obtenidas de GenBank que se utilizaron para elaborar el árbol filogenético.*

Region	Country	GenBank accession number	Isolate
Asia	China	KC679019.1 *Gazella subgutturosa*	Ka1
	China	KC679020.1 *Gazella subgutturosa*	Ka2
	China	KC679021.1 *Gazella subgutturosa*	Ka3
	China	KC679022.1 *Gazella subgutturosa*	Ka4
	China	KC679023.1 *Gazella subgutturosa*	Ka5
	China	KC679024.1 *Gazella subgutturosa*	Ka6
	China	KC679025.1 *Gazella subgutturosa*	Ka7
	China	KC679026.1 *Gazella subgutturosa*	Ka8
	China	KC679027.1 *Gazella subgutturosa*	Ka9
	Pakistan	OK244646.1 *Gazella subgutturosa*	UNGPK085-19
	Pakistan	MH261362.1 *Gazella subgutturosa*	PBRC-GG M4f
	China	KC679008.1 *Procapra picticaudata*	TB1
	China	KC679009.1 *Procapra picticaudata*	TB2
	China	KC679007.1 *Procapra picticaudata*	DL8
	China	KC679006.1 *Procapra picticaudata*	DL7
	China	KC679051.1 *Procapra przewalskii*	SG6
	China	KC679050.1 *Procapra przewalskii*	SG1
	China	KC679049.1 *Procapra przewalskii*	KM3
	Mongolia	KU922905.1 *Procapra gutturosa*	MGL-23
	Mongolia	KU527899.1 *Procapra gutturosa*	MGL-08
	Mongolia	KU527898.1 P*Procapra gutturosa*	MGL-07
	Mongolia	KU527895.1 *Procapra gutturosa*	MGL-04
	China	KC679028.1 *Procapra gutturosa*	IM1
	Mongolia	KX859292.1 *Saiga tatarica*	MGL-162
	China	KC679013.1 *Saiga tatarica*	Saiga4
	China	KF735178.1 *Saiga tatarica*	LYZJ01AA
	China	HQ269461.1 *Pantholops hodgsonii*	m305
	China	KJ862178.1 *Pantholops hodgsonii*	A53
	China	KC679038.1 *Pantholops hodgsonii*	AJ2
	Mongolia	KX859288.1 *Ovis aries*	MGL-152
	China	HQ269453.1 *Capra hircus*	shyjd2
	China	MZ049301.1 *Camelus bactrianus*	Bactrian_camel8-F-coi-1
	Mongolia	KX859287.1 *Bos taurus*	MGL-151
Africa	Egypt	MG571555.1 *Capra hircus*	breed Dimashki
North America	USA	JN850771.1 *Ovis aries*	Sheep_1
	USA	HM102300.1 *Equus caballus*	strain ATCC CCL-57