PMAS
Proceedings of the Mongolian Academy of Sciences
Vol. 65 No 03(255) 2025
https://doi.org/10.5564/pmas.v65i03.4607

ARTICLE

Phylogenetic analysis of mitochondrial cox-1 gene region as a DNA barcode marker for the Gray wolf, Canis lupus in Mongolia

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ARTICLE INFO: Received: 04 Aug, 2025; Accepted: 29 Sep, 2025

Abstract: The gray wolf (Canis lupus), locally known as saaral chono, is a widely distributed species across Europe, North America, and Asia, including Mongolia. Over the last 45 years, Mongolia's gray wolf population has declined threefold, highlighting the need for conservation measures. This study investigates the utility of the mitochondrial cytochrome c oxidase subunit 1 (cox-1) gene as a DNA barcode marker for gray wolves in Mongolia. Tissue samples from ten individuals across ten provinces of Mongolia were collected and sequenced for a region of the cox-1 gene. Phylogenetic analyses show that Mongolian gray wolves cluster with other C. lupus sequences from GenBank, forming two distinct subclades within the species. The cox-1 sequences clearly differentiate gray wolves from other Mongolian Canidae species, demonstrating their value for species identification. These findings support the use of cox-1 as a reliable DNA barcode for monitoring, forensic investigations of illegal hunting, and broader conservation efforts.

Keywords: *Saaral chono, Canis lupus, DNA barcoding, illegal hunting, Near threatened species;*

INTRODUCTION

The gray wolf (*Canis lupus*) is among the most widely distributed terrestrial mammals, occupying diverse habitats across North America, Eurasia, and the Middle East [1,2]. Globally, its population is estimated at ~250,000 individuals and is considered Least Concern according to the IUCN [1] Red List of Threatened Species. In Mongolia, historical population estimates from 1980 suggest ~30,000 individuals, but recent data indicate fewer than 10,000 individuals remaining [2].

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https://doi.org/10.5564/pmas.v65i03.4607

Declines are attributed primarily to habitat degradation, exploitation, persecution, and disease, with regional assessment listing it as Near threatened [3,4]. Mongolia hosts five wild Canidae species, including the *C. lupus*, *Cuon alpinus*, *Nyctereutes procyonoides, Vulpes corsac*, and *Vulpes vulpes* [4]The lifespan of a wolf depends on several factors, including its environment, diet, and threats from other predators or humans. Wolves are highly territorial creatures covering 130–2,600 km² depending on prey availability, making population monitoring challenging. In Mongolia, gray wolves inhabit a range of ecosystems from steppes to forested mountains. The species plays a crucial role in maintaining ecological balance but faces threats from habitat degradation, climate change, and human-wildlife conflicts [1]. Accurate genetic data are essential for understanding population dynamics and implementing effective conservation strategies. Mitochondrial DNA (mtDNA) has long been a tool in evolutionary and ecological studies due to its maternal inheritance, high mutation rate, and lack of recombination [2].

The cytochrome c oxidase subunit 1 (cox-1) gene is widely used as a DNA barcode for species' identification and phylogenetic studies [3]. DNA barcoding, the use of standardized DNA sequences for species identification, was first formally proposed by Hebert (2003)[3] using the mitochondrial cytochrome c oxidase I (COI) gene. In Mongolia, DNA barcoding has been increasingly applied over the past decade to support biodiversity research, conservation, and wildlife management. Initial studies primarily focused on mammals, such as the goitered gazelle (*Gazella subgutturosa*), and Siberian ibex (*Capra sibirica*) where COI sequences were used to clarify population structure and monitor endangered species [4], [5]. Subsequently, DNA barcoding has been applied to other taxa, including also plants, insects, and parasites, and it has supported ecological, such as dietary analysis through metabarcoding. Moreover, forensic applications have emerged, and accordingly Mongolian authorities are using barcoding to identify wildlife products in illegal trade. While these studies demonstrate the growing use of DNA barcoding in Mongolia, many taxa still lack comprehensive reference sequences, highlighting the need for expanded barcoding efforts integrated with conservation and biodiversity monitoring programs. In order 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 [6].

In the present study, we aimed to develop a DNA barcoding system to investigate illegal hunting of *Canis lupus* based on the sequence of the cox-1 gene.

MATERIALS AND METHODS

Sampling locations and DNA extraction

All experiments were approved by the Ethical Committee of National University of Mongolia (approval number: ШУ/У3-2020/02). Tissue samples of ten individuals of *Canis lupus* were collected from 10 *aimags* or provinces in Mongolia (Figure 1, Table 1).The map was generated using ArcGIS 10.4 version (California, United States) in Figure 1.

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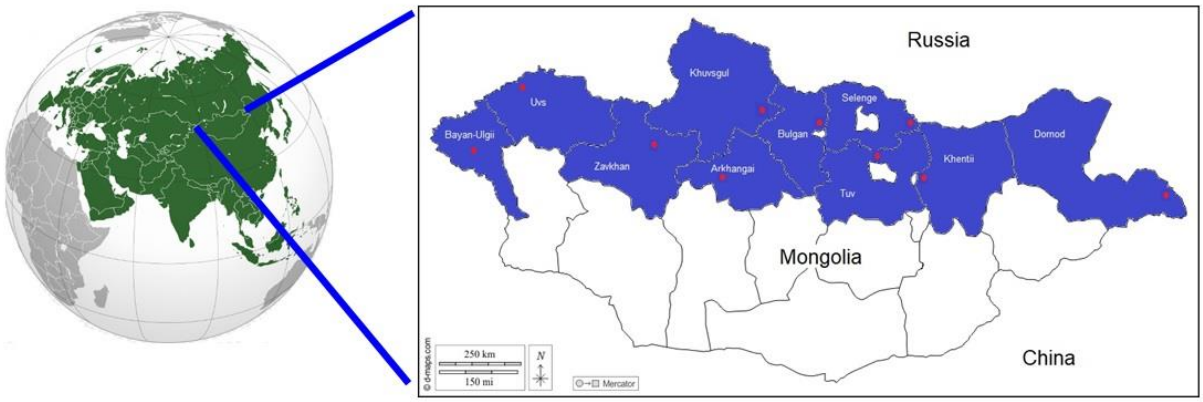

Figure 1. The sampling locations of ten Canis lupus individuals from ten aimags of Bayan-Ulgii, Uvs, Zavkhan, Khuvsgul, Arkhangai, Bulgan, Selenge, Tov, Khentii, and Dornod in Mongolia, Central Asia. One specimen was collected from each province. The red dots on the map indicate the sampling locations. [Source: The map was generated using ArcGIS 10.4 version (California, United States)].

Table 1. Geographic location and Genbank information of samples analysed in this study.

Sequence name	Genbank accession number	DNA barcode sequence size (bp)	Location	Coordinates
MGL-22	**KU922904**	687	Zavkhan, Tosontsengel	*N49.308777739924224 E100.79902094848416*
MGL-29	**KX882036**	640	Selenge, Bugant	*N49.322605022812354 E103.54791954338661*
MGL-30	**KX882037**	633	Bulgan, Khangal	*N49.292908543646504 E104.45473495341089*
MGL-31	**KX882038**	699	Bayan-Ulgii, Buyant	*N47.81426118006061 E92.04196747387684*
MGL-59	**OP236715**	595	Arkhangai, Chuluut	*N47.51875529295324 E99.96479546722131*
MGL-148	**OP236722**	578	Tuv, Batsumber	*N48.35371107596781 E107.0323488903494*
MGL-384	**ON715750**	629	Uvs, Sagil	*N50.29265484142428 E90.88418641797006*
MGL-450	**OP967953**	622	Dornod, Khalkh gol	*N46.7713518594958 E117.50523287204655*
C.l- 1	**KP992982**	516	Khuvsgul, Tarialan	*N50.01262382462312 E102.1408702725457*
C.l- 2	**KP992983**	516	Khentii, Tsenkhermandal	*N47.963151478330815 E109.22964807520798*

Twenty-five-gram tissue samples were collected from animals that died due to snow and extreme cold. The study was done between 2020 and 2025. Most samples were collected in the range of the time in the National institute of forensic sciences and all

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samples were kept in -80 °C degree as fresh tissue samples until used for DNA sequencing. 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 3M acetate Na. The resulting total DNA pellet was dissolved in DNase-free grade water [4], [5].

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 [7], 0.2U Takara DNA polymerase (Kusatsu, Japan), nuclease-free grade water, and the total 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 [7].

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 [7]. The obtained sequences were deposited in GenBank for further use as a DNA barcode marker sequence.

Phylogenetic analysis

The phylogenetic tree of *C. lupus* cox-1 was constructed using Molecular Evolutionary Genetics Analysis version 10.0 (MEGA X) [8]. Additional nucleotide sequences retrieved from GenBank were included in the phylogenetic analysis, and the as accession numbers are listed in Table 2.

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Table 2. Sequences of mitochondrial cytochrome c oxidase subunit 1 gene retrieved from the Genbank.

No.	Country	GenBank accession number	Isolate
1	Mongolia	KU696392.1	*Canis lupus isolate Mongolia1*
2	Mongolia	MN071187.1	*Canis lupus Ms4_Mongolia_wolf*
3	China	PP187786.1	*Canis lupus isolate ST31Dg*
4	African Savanna	*AF028198.1*	*Otocyon megalotis bat-eared fox*
5	North America	*JF443202.1*	*Canis latrans coyote*
6	North Africa	AF028194.1	*Vulpes zerda fennec fox*
7	Arctic	PP939657.1	*Vulpes lagopus Arctic fox*
8	Middle east	MN326069.1	*Vulpes rueppellii Rüppell's fox*
9	Africa	KX012650.1	*Lycaon pictus, african wild dog*
10	Canada, Mongolia	HM380216.1	*Vulpes vulpes, red fox*
11	Russia, Mongolia	MW814846.1	*Nyctereutes procyonoides, raccoon dog*
12	Asia, Mongolia	AF028185.1	*Cuon alpinus, dhole*
13	USA, Mongolia	AY894422.1	*Vulpes corsac*
14	Tibet	NC_027935.1	*Vulpes ferrilata tibetan fox*
15	Brazil	AF028183.1	*Atelocynus microtis, small eared dog*
16	Argentina	*MK321459.1*	*Lycalopex griseus, grey fox*
17	Argentina	MK321443.1	*Speothos venaticus, bush dog*
18	Peru	*AF028187.1*	*Chrysocyon brachyurus, maned wolf*

The exact aligned matrix length was 515 bp. Among the 28 mitochondrial sequences analyzed, we included representatives from the major lineages of the family Canidae, encompassing species from the genera *Canis, Vulpes*, and several other distinct canid groups. These reference sequences were selected to provide a comprehensive phylogenetic framework for comparison, allowing us to verify species, identity and examine the evolutionary placement of Mongolian samples relative to both closely related (*Canis lupus, Vulpes corsac*) and more distantly related canids. This approach ensures that the resulting phylogenetic tree reflects broad canid diversity and provides reliable context for interpreting genetic relationships within and between lineages.

The best model, the Hasegawa-Kishino-Yano (HKY) model for the phylogenetic tree, was determined using the maximum likelihood statistical method [9]. For the outgroup, a sequence of *Nyctereutes procyonoides (Gray, 1834)* (accession number: MW814846.1) was provided. A bootstrap value of 1,000 was set for testing the variants of the phylogenetic tree [10].

RESULTS AND DISCUSSION

In this study, we successfully amplified a specific amplicon of cox-1 with the expected size of 723 base pairs (bp), from 10 DNA samples of *Canis lupus*. After error correction, the sequence lengths ranged from 516 to 699 bp, as listed in Table 1. The cox-1 gene sequences of 10 wolves were tested by pairware sequence comparison using NCBI blast alignment tool between two or more sequences. Query coverage was between 92-100%, and the percentage of Identity was 98.31-100%.

Three more sequences from Genbank were used to confirm the validity of the sequence results. Among the 3 *C.lupus* isolates, 1 was registered from China, and 2 Mongolian *C. lupus* isolates were from different research group [11], [12], [13]. Regarding the *C.lupus* isolates, all 13 isolates (MGL-29, MGL-31, MGL-59, MGL-30,

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MGL-384, MGL-450, MGL-148, MGL-22, CL-1, CL-2, and Mongolia1, Ms4_Mongolia, ST31Dg) including our 10 isolates from 10 *aimags,* clustered as two subclades. Five sequences (KU696392, KU922904, KX882037, ON715750, and PP187786) were genetically closer to each other. Exact locations of Mongolia1 and Ms4_Mongolia are unknown (Figure 2). Three isolates (MGL450, MGL-148, and Ms4_Mongolia) were genetically closer to each other (Figure 2). The remaining 10 isolates, including a Chinese isolate clustered together, forming another subclade within the *Canis* clade. Mongolia and China are geographically close, neighboring countries. The remaining sequences cluster together. The cox-1 sequences clearly separate *C. lupus* from other Mongolian Canidae species (*V. vulpes, V. corsac, C. alpinus, N. procyonoides*), confirming their utility for DNA barcoding.

These results indicate that the cox-1 gene is a reliable genetic marker for identifying gray wolves in Mongolia. It can be incorporated into a DNA Barcoding System to differentiate *C. lupus* from other wildlife species in forensic contexts and support conservation management

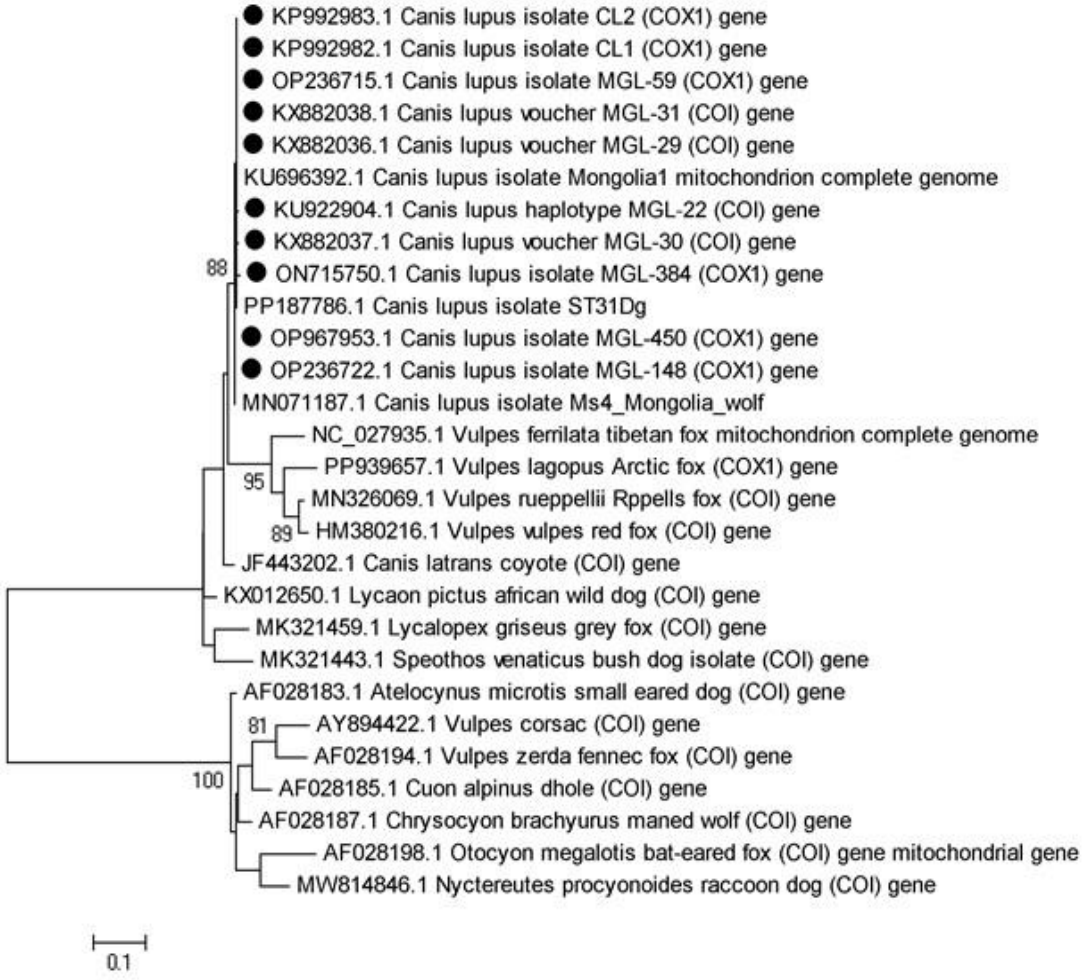

Figure 2. The phylogenetic tree was constructed using partial sequences of cox-1 of the mitochondrial DNA of Canis lupus and other mammalian species. The tree was constructed using the best model, the Hasegawa-Kishino-Yano model. A bootstrap value of 1,000 replications was used for statistical analysis. Values lower than 75% was hidden in the phylogenetic tree. The numbers at the nodes represent statistical support values.

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We have added representative sequences, from different genera of Canidae family. The cox-1 sequence of *Nyctereutes procyonoides* was classified as outgroup, as expected.

Based on our phylogenetic analysis of the cox-1, the *Canis lupus* can be distinguished from Mongolian native wild animal species, particularly from *Vulpes vulpes (Linnaeus, 1758), Vulpes corsac (Linnaeus, 1768), Cuon alpinus (Pallas, 1811), and Nyctereutes procyonoides (Gray, 1834).*

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 *Canis lupus* among other wild animal-derived meat products. Wolves can travel 16 to 48 km in a single day while hunting or patrolling their territory. In areas with scarce prey, they may cover even greater distances. A wolf pack's territory typically ranges from 130 to 2,600 square km, depending on prey availability and competition. So, it is impossible to compare sample origin between provinces or neighboring countries.

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

Acknowledgments

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 of Mongolia. Funders did not have any role in the present study or in the preparation of the manuscript.

Author contributions

The authors confirm contribution to the paper as follows: Study conception and design: BD, BG; data collection: BD, DB, EB; analysis and interpretation of the results: BU, BG; draft manuscript preparation: BD, EB. All authors reviewed the results and approved the final version of the article.

Source of funding

This work was supported by the Mongolian Science and Technology Foundation (approval number: ШУ/УЗ-2020/02).

Conflicts of interest

The authors declare that there is no conflict of interest.

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