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Molecular Identification of Canis Lupus by Coi Gene from Sargodha District, Punjab

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Abstract

Advancements in molecular biology have significantly improved the identification of organisms. Molecular identification uses genetic information to distinguish species with high accuracy. Among the various molecular tools available, the cytochrome c oxidase subunit I (CO1) gene, found in the mitochondrial genome, has emerged as one of the most reliable markers for animal identification. It combines a conserved sequence structure with a relatively high mutation rate, making it ideal for distinguishing even closely related species. This study aims to address these limitations by employing DNA barcoding using the Cytochrome c oxidase I (CO1) gene, a universally accepted molecular marker for species-level identification in animals. In this study, DNA samples were be collected from Bully Dogs across multiple localities in the Sargodha District. The CO1 was amplified, sequenced, and compared against global reference databases, particularly the Barcode of Life Data System (BOLD), to identify distinct genetic signatures and variations. This molecular approach is intended to provide a more accurate and reliable method for breed verification by revealing intra- and inter-breed genetic differences. The anticipated findings shed light on the genetic diversity and evolutionary relationships of local Bully Dogs with other members of the Canis lupus species. The results are expected to have practical implications for breed conservation, sustainable breeding programs, and the prevention of inbreeding-related issues. Furthermore, this study offers potential applications in wildlife forensics, enhancing the regulatory framework for breed certification, and curbing illegal breeding practices. By establishing a genetic baseline for Bully Dogs in the region, this research contributes to preserving their genetic integrity and sets a precedent for the use of molecular tools in domestic animal classification and conservation strategies.

Keywords: Cytochrome c oxidase I, Canis lupus (Bully Dog), Barcode of Life Data System (BOLD), Molecular Identification, Genetic conservation.

Introduction

Modern advancements in molecular biology have significantly improved the identification and classification of organisms. Unlike traditional morphological techniques, molecular identification uses genetic information to distinguish species with high accuracy. This method has become invaluable in fields such as conservation biology, evolutionary studies, and forensic science [1]. Among the various molecular tools available, the cytochrome c oxidase subunit I (CO1) gene, found in the mitochondrial genome, has emerged as one of the most

reliable markers for animal identification. It combines a conserved sequence structure with a relatively high mutation rate, making it ideal for distinguishing even closely related species [2,3].

The genus *Canis* includes species such as wolves (*Canis lupus*), domestic dogs (*Canis lupus familiaris*), coyotes, and jackals. These species often share overlapping morphological features and genetic similarities, making them difficult to distinguish based solely on physical traits. This complexity is especially pronounced in the gray wolf, which is ecologically significant due to its wide distribution and role as a top predator [4]. Accurate identification is essential for managing endangered subspecies, such as the Mexican gray wolf (*Canis lupus baileyi*) [5]. The use of molecular markers like CO1 allows for precise species discrimination, tracking of population dynamics, and detection of hybridization with domestic dogs, which poses a threat to genetic integrity [6,7].

The CO1 gene plays a vital role in cellular respiration as part of the mitochondrial electron transport chain. Its effectiveness as a molecular marker stems from several features of mitochondrial DNA: maternal inheritance, lack of recombination, and a predictable mutation rate [1,8]. Hebert et al. were the first to propose CO1 as a universal DNA barcode for animals, and subsequent research has confirmed its utility across a wide range of taxa [1,2]. In *Canis* species, CO1 has been instrumental in distinguishing wolves from domestic dogs and in identifying hybrid individuals, thereby aiding in conservation efforts [6,9].

Molecular identification using the CO1 gene follows a series of standard laboratory procedures. First, DNA is extracted from samples such as blood, hair, or tissue. Polymerase Chain Reaction (PCR) is then used to amplify the CO1 gene, generating sufficient quantities of the target DNA sequence [10]. The amplified sequences are then analyzed and compared to reference databases such as GenBank and the Barcode of Life Data System (BOLD) [2,11]. A divergence of approximately 2% or more between sequences typically indicates interspecific variation, while lower divergence often suggests variation within the same species [1].

The CO1 gene has numerous practical applications in both conservation and forensic contexts. In conservation biology, it helps assess genetic diversity within wolf populations, guide breeding programs, and evaluate the success of reintroduction initiatives [5,8]. CO1 barcoding also assists in detecting hybridization between wolves and domestic dogs, which is crucial for preserving the genetic integrity of wild populations [6,13]. In forensic science, CO1-based barcoding is used to identify species in cases of poaching, illegal wildlife trade, and livestock predation, thereby supporting legal enforcement and wildlife protection efforts [14,15].

The Canis lupus, a species of wolf, is a key focus of research in the field of animal taxonomy. The CO1 gene, a mitochondrial DNA marker, is a key tool for precise species identification. However, there is a need for more region-specific studies to explore its effectiveness in differentiating closely related Canis species. Species Genetic Differentiation (SGD) analysis using CO1 gene sequences can provide valuable insights into genetic distances and relationships among Canis populations, but there is limited research on Canis lupus molecular identification in certain regions. This study aims to address these gaps by applying CO1-based molecular techniques and SGD analysis to accurately identify Canis lupus and assess its genetic distinction from related species

DNA barcoding using the cytochrome c oxidase subunit I (CO1) gene has transformed the way scientists identify and differentiate species. This molecular method analyzes a short, standardized DNA region from the mitochondrial genome, offering a reliable and precise alternative to traditional classification methods that depend on physical appearance. In contrast, CO1 barcoding provides species-level resolution based on genetic data that is consistent across individuals of the same species (21).

The CO1 gene has become the gold standard for animal barcoding because it combines conserved regions—allowing for easy amplification—with variable sites that show enough

divergence to distinguish between species. For example: • Taxonomy and Systematics: DNA barcoding simplifies species classification, including cryptic species that are difficult to identify morphologically. • Conservation Biology: CO1 barcoding helps in the identification of endangered or vulnerable species and provides data for developing species-specific conservation strategies. • Environmental Monitoring: Through techniques like eDNA analysis, CO1 can detect organisms from water, soil, or air samples, allowing for non-invasive biodiversity assessments. Moreover, large-scale international initiatives such as the Barcode of Life Data System (BOLD) and the International Barcode of Life (iBOL) have incorporated CO1 sequencing into global biodiversity databases. These platforms allow scientists to match unknown DNA sequences to reference species, streamlining the identification process and enabling collaborative biodiversity research worldwide (23)

The aims of the present work; to evaluate the effectiveness of the **Cytochrome c Oxidase I (COI) gene** in the genetic identification of *Canis lupus*, to explore the potential of **DNA barcoding** as a tool for species-level identification and classification within the *Canis lupus* populations, and to contribute to the **molecular taxonomy and conservation genetics** of *Canis lupus* through comparative mitochondrial analysis.

MATERIALS AND METHODS

Advantages Over Traditional Methods

Traditional ways of identifying species often depend on visible characteristics like shape, size, color, or patterns. Also, in many cases, researchers only have access to incomplete samples, such as fossils, fragments, or early life stages like larvae, which may not have the necessary features for accurate identification. DNA barcoding, especially using the CO1 gene, offers a solution to these problems by relying on genetic information rather than physical appearance. One of the biggest advantages is that it can be used at any life stage—from eggs and larvae to adults—even when the sample is preserved or degraded. It also opens doors for environmental studies through eDNA, where scientists can identify species just by analyzing DNA left behind in the environment, like in water, soil, or air samples. In short, DNA barcoding is not only more accurate and efficient, but also far more flexible and powerful than traditional methods, especially in complex or challenging identification scenarios.

Sampling detail

The table 1 shows the samples of Canis lupus (Bully dog) collected on various dates from different locations. Sample IDs range from CL01 to CL05, with collection dates spanning from September 25, 2024, in Shaheenabad to November 11, 2024, in Bhalwal. The samples were gathered from Shahpur city, Shahpursadar, Khushab city, and Bhalwal, providing a geographical distribution overview for the study.

Procedure

DNA Extraction and PCR Amplification Step

Sample Collection and Storage

• Blood samples were collected in EDTA tubes to prevent coagulation.

• Samples were immediately stored at freezing temperatures to preserve DNA integrity.

Preparation of Lysis Solution

• A lysis buffer was prepared with the following composition: o 0. 5) o 5 mM $MgCl_2$ o 1% Triton X-100 • This buffer was used to break open the blood cells and begin the DNA release process.

Lysis and Digestion

330 µl of lysis solution was added to the blood cell mixture. • The sample was thoroughly mixed using a vortex machine to ensure even distribution of reagents.

Incubation for Cell Lysis
The sample was placed in a water bath at 56°C overnight (~16 hours) to facilitate lysis and digestion. • After overnight incubation: o The tube was vortexed again to resuspend the contents.
3 Polymerase Chain Reaction (PCR). Following successful extraction, the mitochondrial cytochrome c oxidase I (CO1) gene was amplified using Polymerase Chain Reaction (PCR). The PCR process involved three main thermal cycling.

Gene Sequencing and Database Comparison
Once the CO1 gene was amplified, it was subjected to DNA sequencing. Depending on the scale of analysis, either Sanger sequencing or next-generation sequencing (NGS) was employed. Sanger sequencing was used for high-precision, single-sample analysis, while NGS was reserved for large-scale, high-throughput sequencing needs.
The resulting DNA sequences were then compared against reference databases such as GenBank and the Barcode of Life Database (BOLD). These platforms provide extensive, curated collections of DNA barcodes, enabling accurate species identification based on genetic similarity

RESULTS
DNA Extraction and quantification
Genomic DNA OF 5 Blood samples were examined by gel electrophoresis for quantification and confirmation.1% agarose was used in this process. DNA extraction was done by Russel and Sambrook method with some modifications according to the need.The whole process is carefully done under the specific concentration of protein kinase K and phenol chloroform.

Polymerase chain reaction
For the amplification of desired region COI, the polymerase chain reaction was performed from the extracted DNA.1000 base pairs was amplified by using universal primer pair for analysis of sample products of Canis **lupus (Bully dog)** are given below (figure 1):

Sequence analysis
Sequence Editing and Alignment
After successful amplification and sequencing of the CO1 gene, the raw DNA sequences were first examined and refined using **BioEdit v7.0.5**, a widely used manual sequence editing software designed for precise proofreading and cleanup of nucleotide sequences. This software allows researchers to detect and correct errors such as ambiguous bases, gaps, and reading frame disruptions in the sequences prior to alignment, ensuring high-quality data for downstream analyses (33).
Sequence Alignment
Once the sequences were curated, **multiple sequence alignment** was performed using **ClustalW,** an established algorithm integrated into the **MEGA X (Molecular Evolutionary Genetics Analysis Version X)** software package. MEGA X provides robust tools for aligning sequences, estimating evolutionary distances, and constructing phylogenetic trees. ClustalW facilitates the identification of conserved and variable regions across sequences, which is essential for assessing genetic relationships and species delimitation (34)
Similarity Search and Species Confirmation
To further validate and identify the species origin of the samples, the aligned sequences were submitted to the **BLAST (Basic Local Alignment Search Tool)** platform. This tool compares

the input sequence against the **NCBI GenBank** database to find highly similar sequences and determine the closest known relatives. Through BLAST analysis, researchers can accurately infer species identity by evaluating sequence similarity scores and alignment coverage (35).

Reference Data from GenBank

For comparative analysis, reference sequences of the **cytochrome c oxidase subunit I (CO1) gene** from recognized species, including *Canis lupus*, were retrieved from the GenBank database. These reference sequences serve as benchmarks for evaluating the distinctiveness and taxonomic placement of the test samples. They provide a reliable genetic background to differentiate between closely related or morphologically similar species (36).

Intraspecific and Interspecific Divergence Analysis

To understand the genetic variability within and between species, the sequences were subjected to analytical tools that calculate **intraspecific (within species)** and **interspecific (between species)** divergence. These tools assess the nucleotide differences and generate statistical values that help in distinguishing between species. A low intraspecific divergence combined with a high interspecific divergence supports the validity of species boundaries, which is crucial for DNA barcoding and phylogenetic studies (37).

Maximum Likelihood Estimate of Gamma Parameter for Site

The table 2 shows the genetic distances between different nucleotides A, T, C, and G. The values in the table represent the genetic distance between the nucleotides in some way. The diagonal values are typically zeros as the distance from a nucleotide to itself is zero. The other values show the genetic distances between different nucleotide pairs. For example, the distance between A and T is 5.1672, between A and C is 4.9028, between A and G is 13.1738, and so on. This type of table is often used in bioinformatics and genetics to analyze genetic relationships and evolutionary distances.

The estimated value of the shape parameter for the discrete Gamma Distribution is 5.8320. Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+G) [1]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+G]). Mean evolutionary rates in these categories were 0.50, 0.75, 0.94, 1.17, 1.63 substitutions per site. The nucleotide frequencies are A = 27.07%, T/U = 25.64%, C = 24.33%, and G = 22.96%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -14275.515. This analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1901 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

Each entry is the probability of substitution (r) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model [1]. Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in italics. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of rvalues is made equal to 100, The nucleotide frequencies are A = 27.07%, T/U = 25.64%, C = 24.33%, and G = 22.96%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -14298.813. This analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1901 positions in the final dataset. Evolutionary analyses were conductedinMEGA11[2)

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Maximum Parsimony analysis of taxa

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 4188 is shown. The consistency index is 0.606495 (0.500455), the retention index is 0.365422 (0.365422), and the composite index is 0.221626 (0.182877) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. [1]) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1901 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2] (Table 3; Figure 3). The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model [1]. This analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1901 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

DISCUSSION

Genetic markers, particularly mitochondrial DNA markers like the Cytochrome c Oxidase I (COI) gene, have revolutionized how species are identified, genetic diversity is assessed, and evolutionary relationships are understood. The COI gene has become a cornerstone in molecular taxonomy and species differentiation due to its high mutation rate and species-specific sequence variations, which make it especially effective in identifying cryptic species and distinguishing closely related taxa (37). This gene's ability to provide high-resolution genetic data has made it invaluable for DNA barcoding, a powerful tool that enables researchers to identify species with greater accuracy than traditional morphological methods.

In the context of *Canis lupus*, the gray wolf, COI has been extensively utilized to explore its genetic diversity, population structure, and evolutionary history. Using mitochondrial markers like COI, researchers have successfully differentiated between various populations and subspecies of wolves, which is crucial for conservation efforts (38). The COI gene has demonstrated a high level of efficiency in identifying genetic variation among wolf populations, which is essential for strategies aimed at protecting these populations and ensuring their survival (39). This use of COI to resolve identification challenges and enhance genetic assessments reflects the gene's central role in modern wildlife biology.

One of the primary advantages of COI over nuclear DNA markers is its higher mutation rate, which provides a more detailed insight into genetic divergence and phylogenetic relationships. In *Canis lupus*, sequencing of the COI gene has been used to track evolutionary lineages and migration patterns, offering valuable information that is essential for species management and conservation strategies (40). The ability to accurately determine genetic divergence between wolf populations helps in identifying distinct evolutionary lineages, making COI a crucial tool for effective population management (41).

Phylogenetic studies that incorporate COI sequences have been key in constructing evolutionary trees that help researchers understand population dynamics, genetic health, and the evolutionary processes that have shaped wolf populations. These studies have also shown how the comparison of COI sequences across different populations can highlight significant

genetic differences and reveal adaptive traits that have evolved in response to environmental pressures (42). Such insights are essential for informing conservation decisions, especially when certain populations may be genetically distinct or face threats from habitat loss or human-induced changes.

While the Cytochrome b gene (CYTB) has been widely used for mitochondrial studies in birds, the COI gene is often preferred for species differentiation because of its broader reference database and higher interspecific variability (43). In *Canis lupus*, the COI gene has been instrumental in identifying hybridization events, particularly those involving domestic dogs or other canid species. These hybridization events are a concern for conservation efforts because they can compromise the genetic integrity of pure wolf populations, making COI an essential tool for detecting and monitoring these events (44).

By integrating COI-based molecular analysis with other mitochondrial DNA data, scientists can gain a more holistic understanding of genetic diversity within *Canis lupus* populations. Combining COI data with information from other markers allows for a deeper understanding of evolutionary history, population dynamics, and the genetic relationships between different wolf populations. This integrated approach not only helps in identifying genetically distinct populations but also contributes to more effective conservation management, helping to prevent inbreeding and other genetic issues that could affect population health.

Future studies on *Canis lupus* should focus on sequencing the COI gene in different populations and comparing these sequences with global reference databases. This will enable researchers to validate species identity, confirm evolutionary relationships, and refine taxonomic classifications. By expanding the COI sequence database and applying it to a wider range of wolf populations, scientists better equipped to address conservation challenges and ensure that these iconic species continue to thrive in the wild.

In summary, the COI gene is an invaluable tool for studying the genetic diversity and evolutionary relationships of *Canis lupus*. Its application in molecular taxonomy and its ability to provide high-resolution genetic data make it a central marker for modern wildlife research. As we continue to develop and refine COI-based methodologies, we can improve conservation strategies, better manage wolf populations, and ultimately contribute to the long-term survival of *Canis lupus*.

Conclusion

This study highlights the significant role of the Cytochrome c Oxidase I (COI) gene in molecular taxonomy, particularly in understanding the genetic diversity and evolutionary history of Canis lupus. In particular, COI-based investigations have helped track migration patterns, identify hybridization events with domestic dogs, and reveal the evolutionary pathways that Canis lupus has taken across different habitats. Looking forward, the continued use of COI sequencing in Canis lupus studies can enhance global databases, improve species verification, and support conservation programs aimed at mitigating population decline. In essence, the COI gene is more than just a barcode—it is a window into the genetic identity and evolutionary journey of Canis lupus, offering vital information that helps bridge the gap between genetic science and practical conservation.

Supplementary materials

All the tables and figures are given below.

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