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Mitochondrial DNA analysis of Hassawi donkeys reveals genetic diversity and global maternal lineages

Faisal Almathen[1,2]*

[1]Department of Veterinary Public Health, College of Veterinary Medicine and Animal Resources, King Faisal University, 400 Al-Hasa, Saudi Arabia.
[2]Camel Research Center, King Faisal University, P.O. Box 400, Al Hufuf 31982, Al-Ahsa, Saudi Arabia.

Corresponding author: Faisal Almathen
E-mail: falmathen@kfu.edu.sa.sa

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ABSTRACT. The Hassawi donkey, an indigenous breed from Saudi Arabia, has adapted to the arid climate of the region and exhibits distinct physical traits. However, limited research has been conducted on its genetic diversity and evolutionary history. This study investigates the genetic diversity and maternal lineage of the Hassawi donkey by analyzing the mitochondrial DNA Cytochrome b (Cytb) gene from 21 individuals. Using polymerase chain reaction (PCR) and high-fidelity sequencing, we identified three single nucleotide polymorphisms (SNPs) at positions 14289, 14409, and 14424, which differentiated two distinct haplotypes within the population. Phylogenetic analysis using the Neighbor-Joining (NJ) method revealed two major clusters corresponding to these haplotypes. The first haplotype, representing five individuals, aligned with Haplogroup A, while the second haplotype, encompassing 16 individuals (76% of the samples), aligned with Haplogroup B. These findings were further supported by a median-joining network analysis that compared the Hassawi donkeys to global populations, including samples from Africa and Asia. This analysis confirmed that the Hassawi donkeys share maternal ancestry with global donkey populations, particularly those from Africa and Asia.

The results provide new insights into the genetic diversity of the Hassawi donkey, revealing its connection to global donkey populations and contributing to the broader understanding of donkey domestication and evolution. This study represents the first genetic analysis of the Hassawi donkey and provides a foundation for future research on its genetic structure and conservation.

Key words: Genetic diversity; donkey; Cytochrome b gene; polymorphism; haplotypes; phylogenetic

INTRODUCTION

The global donkey population, estimated at over 50.5 million individuals, is spread across diverse continents and countries, with China and Ethiopia recognized as key nations involved in donkey breeding (https://www.thebrooke.org/our-work/data-working-equids). However, in mechanized agricultural sectors, particularly in Europe and the Americas, donkey populations have declined sharply, leading to the disappearance of some breeds and the threat of extinction for others (Quaresma et al., 2014). The genetic diversity of donkeys has been a focal point in many studies, particularly through the use of mitochondrial DNA (mtDNA). mtDNA, inherited solely through the maternal line, has several characteristics—such as a high mutation rate and large copy number—that make it a valuable tool for investigating phylogenetic relationships, domestication events, and genetic diversity in donkeys (Han et al., 2014; Ma et al., 2020; Wang et al., 2022; Xia et al., 2023). This marker allows researchers to explore maternal lineages and trace evolutionary patterns across different geographic regions (Chen et al., 2009; Kefena et al., 2014; Matassino et al., 2014; Cinar Kul et al., 2016; Cozzi et al., 2018; Ünal et al., 2010).

Previous genetic and archaeological studies strongly support an African origin for domestic donkeys (Beja-Pereira et al., 2004; Rossel et al., 2008; Kefena et al., 2014; Wang et al., 2020; Todd et al., 2022). The "pastoralist hypothesis" suggests that donkeys were domesticated between 7,500 and 6,500 years ago in the northeast African grasslands, in response to changing climatic conditions, such as droughts and increasing aridity, which encouraged human populations to adopt a more mobile lifestyle (Fletcher 1999).

Mitochondrial DNA studies have identified two distinct maternal lineages—Clade 1 and Clade 2 suggesting two separate domestication events. Clade 1 is believed to have originated from the Nubian wild ass (*Equus africanus africanus*), while the origin of Clade 2 remains unclear, though it is unlikely to have come from the extinct Somali wild ass (*Equus africanus somaliensis*) (Beja-Pereira et al., 2004). Whole genome sequencing has confirmed that modern domestic donkeys originated from a single African population, encompassing both Clades 1 and 2 (Pereira et al., 2004; Aranguren et al., 2004). Furthermore, Rambaldi Migliore et al., (2024) identified two primary haplogroups, A and B, within the global donkey population.

Although donkeys have been raised for generations in Saudi Arabia, particularly the Hassawi breed from the Al-Ahsa region, little genetic research has been conducted on this population. Existing studies have focused mainly on the breed's physical, hematological, and biochemical characteristics. Shawaf et al., (2017) described the Hassawi breed as a giant donkey breed with significant differences in chest width and depth between males and females (Shawaf 2017). Another study by Shawaf et al., (2017). found unique hematological and biochemical traits in the breed, which were thought to be influenced by a diet rich in dates (Shawaf et al., 2017).

Despite the utility of mtDNA in studying donkey phylogenetics, no genetic study has yet been conducted on Hassawi donkeys in Saudi Arabia. This study aims to address this gap by evaluating the genetic diversity and origins of the Hassawi breed through the sequencing and analysis of the mitochondrial Cytochrome b (Cytb) gene. By comparing the Cytb sequences of Hassawi donkeys to global populations, this study seeks to uncover genetic variations, identify haplotypes, and explore the maternal lineage of this unique donkey breed.

MATERIALS AND METHODS

Samples and DNA Extraction

A cohort of twenty-one Hassawi donkeys was strategically selected to represent the genetic diversity of the Hassawi donkey population from the Al-Ahsa region of Saudi Arabia. Blood samples (5 ml) were collected from each individual for genomic analysis. DNA extraction was performed using the DNeasy® Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. The concentration and purity of the extracted DNA were measured using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific™). All DNA samples were stored at -80°C for further analysis. The study was conducted according to the guidelines of the Animal Ethics Research Committee at King Faisal University.

PCR amplification and sequencing

The complete mitochondrial DNA Cytb gene was amplified using specific primers as described in a previous study (Sun et al., 2016). The PCR reaction mixture contained 12.5 µl of Phusion High-Fidelity PCR Master Mix (Thermo), 10 picomoles of each primer, 2 µl of genomic DNA template, and nuclease-free water to bring the total volume to 25 µl. The thermal cycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for 10 minutes. The amplified products were visualized on a 1% agarose gel using the Bio-Rad Gel Doc XR Imaging System. PCR products were stored at -80°C until sequencing, which was carried out at Macrogen's facilities in South Korea.

Sequence quality assessment

Sequence quality was assessed by evaluating both Phred scores and chromatogram peaks using BioEdit v7.7 (Hall et al., 1999). Low-quality regions at the sequence ends were trimmed, and only high-quality bases were retained for further analysis. A consensus sequence of 900 bp was generated from the 21 samples. The consensus sequences were aligned using the MUSCLE algorithm in MEGA11 and compared to the NCBI Reference Sequence for the mitochondrial genome of *Equus asinus* (NC_001788.1). Any discrepancies or ambiguous bases were manually reviewed and resolved.

Phylogenetic analysis and median-ioining network

Phylogenetic relationships were first explored by constructing a Neighbor-Joining (NJ) tree using the HKY substitution model with a bootstrap value of 1000, based on selected sequences from GenBank.

Following the phylogenetic analysis, a median-joining network was constructed using DnaSP v6 (Rozas et al., 2017) and PopART v1.7 (Leigh and Bryant, 2015) for the Hassawi donkey samples. These sequences represented various countries and included known haplogroups A and B. Global haplotype network analysis was conducted using MEGA v11 (Tamura et al., 2021) to illustrate the relationships between identified haplotypes and their geographic distributions. Haplotype sequences generated in this study were deposited in GenBank under accession numbers OR498910-OR498911

RESULTS

Gene variations and haplotype analysis

A comparative analysis of the mitochondrial DNA Cytochrome b (Cytb) gene in 21 Hassawi donkeys revealed the presence of two distinct haplotypes, which were differentiated by three nucleotide variations. These variations occurred at positions 14289, 14409, and 14424 when aligned against the reference mitochondrial genome (NC_001788). Specifically, sequences classified as Haplotype 1 displayed a transition from C to T at all three positions, while sequences in Haplotype 2 showed no variation relative to the reference, as outlined in Table 1. The genetic differences observed between these haplotypes were exclusively attributable to single nucleotide polymorphisms (SNPs), with no insertions, deletions, or transversions detected.

Phylogenetic relationships and median-joining network analysis

Phylogenetic analysis using the Neighbor-Joining (NJ) method further validated the haplotype distinction, dividing the Hassawi donkey sequences into two distinct clusters. Haplotype 1, comprising five sequences (Hassawi #2, #12, #24, OR498909, and OR498910), was represented by a red branch in the NJ tree (Figure 1). Haplotype 2, consisting of 16 sequences, was illustrated in green. These clusters clearly delineated the two genetic groups within the sample population.

To explore the relationship between the Hassawi donkeys and global populations, a median-joining network analysis was conducted using sequences from Saudi Arabia, China, Turkey, Kenya, Nigeria, Kyrgyzstan, and Tajikistan. This analysis demonstrated that the five sequences in Haplotype 1 were associated with Haplogroup A, while the 16 sequences in Haplotype 2 corresponded to Haplogroup B, as shown in Figure 2. The consistency between the NJ tree and the median-joining network confirms the genetic differentiation between these groups and their alignment with known global haplogroups.

DISCUSSION

The genetic exploration of the Hassawi donkey, an indigenous breed from Saudi Arabia, offers vital insights into its domestication history and maternal lineage. Known for its adaptability

Table 1. Comparison of nucleotide variations in cytochrome b gene positions between Hasswai donkey haplotypes/ haplogroups and the reference mitochondrial genome.

Position	Reference_NC001788	Haplotype 1/Haplogroup A	Haplotype 2/ Haplogroup B
14289	C	T	C
14409	C	T	C
14424	T	C	T

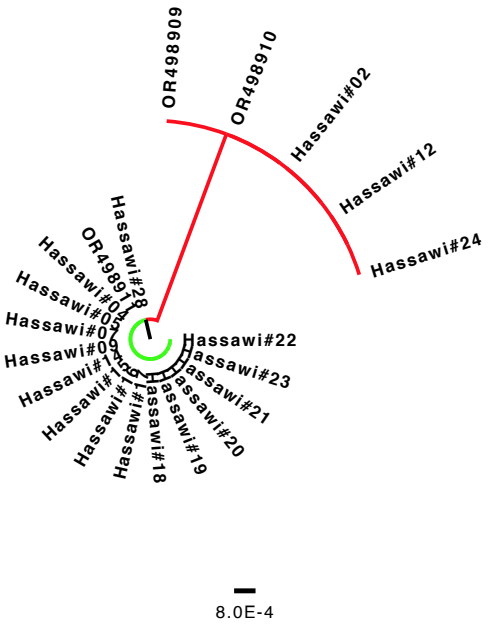

Figure 1. Neighbor-Joining (NJ) phylogenetic tree illustrating the genetic relationships among 21 Hassawi donkeys based on mitochondrial Cytochrome b (Cytb) gene sequences. The tree was constructed using the HKY substitution model with 1000 bootstrap replicates for branch validation. The samples are clustered into two distinct haplotypes, with bootstrap values shown along the branches, indicating the robustness of each clade. The samples include both labeled individuals from the Hassawi population (Hassawi#) and representative sequences submitted to GenBank (OR498909, OR498910, and OR498911). The scale bar represents the genetic distance calculated as substitutions per site.

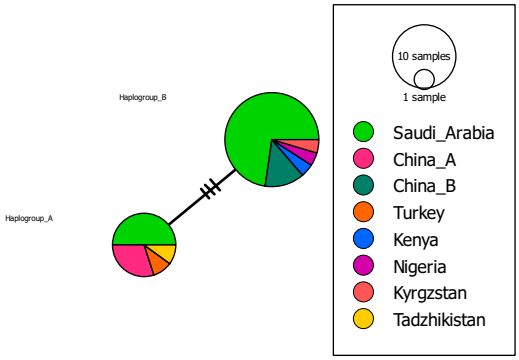

Figure 2. Median-Joining (MJ) network of Hassawi donkeys based on mitochondrial Cytochrome b (Cytb) gene sequences, depicting their relationship within two known global haplogroups, A and B. The network was constructed using sequences from different geographic regions, including Saudi Arabia, China, Turkey, Kenya, Nigeria, Kyrgyzstan, and Tajikistan. The Hassawi donkey haplotypes are distributed between both Haplogroup A and Haplogroup B, with the majority of the samples clustering in Haplogroup B, reflecting the genetic diversity within the population. The size of each node is proportional to the number of samples sharing that haplotype.

to challenging terrains, particularly in arid regions, the Hassawi donkey is a valuable subject in evolutionary studies. Despite its physical distinctiveness—such as long ears, large eyes, and a narrow head—there has been little prior genetic research on this breed, making this study an important contribution to understanding its genetic diversity and origins.

By sequencing the mitochondrial DNA Cytochrome b (Cytb) gene from 21 individuals of the Hassawi breed, this study provides a comprehensive view of its genetic landscape and connections with global donkey populations. The identification of three single nucleotide polymorphisms (SNPs) at positions 14289, 14409, and 14424 within the Cytb gene underscores the presence of genetic diversity within the Hassawi breed. These polymorphisms are key markers of variation when compared to the reference genome (*NC_001788*). The absence of insertions, deletions, or transversions further reinforces the stability of the mitochondrial genome, with the variations confined solely to SNPs.

The haplotype analysis revealed two distinct haplotypes in the Hassawi population. Haplotype 1 was found in five individuals, while Haplotype 2, more prevalent, encompassed 16 individuals, accounting for 76% of the samples. Haplotype 2's association with Haplogroup B indicates that the majority of the Hassawi donkeys share a maternal lineage with donkey populations worldwide, consistent with earlier findings on the widespread presence of Haplogroup B in global donkey populations (Zhang et al., 2010; Stanisic et al., 2017; Cozzi et al., 2018; Aranguren et al., 2004; Yun et al., 2022). This suggests that the Hassawi breed is part of a broader maternal lineage that includes both African and Asian donkeys.

Phylogenetic analysis using the Neighbor-Joining (NJ) method provided additional validation for the haplotype distinction, clearly separating the samples into two distinct clusters. Haplotype 1, comprising a smaller subset, was represented by a red branch in the NJ tree, while Haplotype 2, representing the majority of the samples. This clear clustering highlights genetic divergence within the Hassawi population, which may reflect distinct maternal lineages or historical breeding patterns.

The median-joining network further contextualized the relationship of the Hassawi donkeys with global populations. By incorporating sequences from countries such as Saudi Arabia, China, Turkey, Kenya, Nigeria, Kyrgyzstan, and Tajikistan, the network analysis confirmed the presence of two main haplogroups—A and B—in the Hassawi breed. The alignment of Haplotype 1 with Haplogroup A and Haplotype 2 with Haplogroup B suggests that the Hassawi donkeys are part of the same global mitochondrial lineage as other domestic donkeys. This finding aligns with the "pastoralist hypothesis" that donkeys were domesticated in northeast Africa between 7,500 and 6,500 years ago in response to changing climatic conditions (Fletcher 1999).

The evolutionary divergence analysis revealed significant genetic differentiation within the Hassawi population, as demonstrated by the clustering of samples into two groups. This genetic complexity, illustrated by both the NJ tree and the median-joining network, mirrors the broader evolutionary history of donkeys, which have been shaped by domestication events and geographic spread. The connection of the Hassawi donkeys with both African and Asian donkey populations further emphasizes the breed's unique genetic makeup, while also highlighting its shared heritage with donkey populations around the world.

In conclusion, this study provides the first genetic insight into the Hassawi donkey population through the analysis of mitochondrial DNA. The identification of two haplotypes and their alignment with global haplogroups offers a window into the evolutionary history of the breed. These findings fill a critical gap in our understanding of the genetic diversity and maternal lineage of the Hassawi

donkey and underscore the breed's link to global donkey populations. Future research incorporating nuclear DNA or whole-genome sequencing would offer a more comprehensive picture of the genetic structure and historical evolution of the Hassawi breed.

ABBREVIATIONS

Cytb: Cytochrome b

mtDNA: mitochondrial DNA

SNPs: single nucleotide polymorphisms

AUTHOR CONTRIBUTIONS

FA Conceptualization, Data Curation, Methodology, writing—original draft preparation, review and editing, supervision, project administration and funding acquisition.

FUNDING

No funding was received for this study.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study is publicly available in the GenBank accession no. OR498910-OR498911

DECLARATIONS

Institutional Review Board Statement: The study was performed as per the guidelines and Animal Ethics Research Committee approval at the King Faisal University.

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CONFLICTS OF INTEREST

The author declares no conflict of interest.

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