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# Mitochondrial DNA Variation and Genetic Relationships in Indian Halari Donkey Breed using D-Loop Region

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## ABSTRACT

**Background:** Halari donkey is a distinct breed of India that comes from the community of migrants from the regions of Halar, Bharwad and Rabaris of Gujarat. They have a brief history of travel and migration from arid and semi-arid regions of India. The population of Halari donkeys has fallen drastically in past and is further declining; therefore, these donkeys may be recognized as a threatened breed in near future.

**Methods:** The genetic diversity of this breed was determined using the D-loop gene of 542 bp size belonging to mitochondrial DNA and compared with the complete mitochondrial genome of *Equus asinus* (NC_001788.1). In the present study, a total of 8 haplotypes were identified with haplotype diversity of 0.8152 and nucleotide diversity of 0.1281 indicating that the population has high genetic diversity. The Median-joining network tree and neighbour-joining trees revealed close clustering of the Halari donkeys.

**Result:** The high nucleotide and haplotype diversity suggest Halari donkeys as a unique population and proper management and conservation is the need of the hour.

**Key words:** Breed, Donkey, Genetic diversity, Halari, Mitochondria.

## INTRODUCTION

India is the land of vast farm animal genetic resources which play an important role in civilization along with economic importance. It is necessary to understand their breeding techniques and genetic explorations to create better development processes and a sustainable ecosystem. Such studies will help in raising animals that provide better yield along with diverse genetic characteristics. However, it can only be possible when there is a complete understanding of the genetic data related to the animals that we are dealing with. In India, there has been a drastic reduction in the population of Indian equines (Bhat *et al*., 1981) along with other livestock animals (Madhusudhan *et al*., 2004). By the year 2019, the population of equines was reduced from 1.14 million to 0.54 million (20th livestock census, 2019). However, these farm animals have the highest level of genetic variations due to their breeding differences as well as having more species (Groeneveld *et al*., 2010).

The Donkeys, *Equus asinus* are domesticated equines that have originated around 55 million years ago (Woodburne *et al*., 1994; Schubert *et al.,* 2014). They have been widely used for domestication as pack animals as well as for transportation throughout the years causing their migration across the world. They were also used for lifting heavy weights on mountain ranges due to their high tolerance capacities. Halari donkey is one of the significant breeds of equine exclusive to India that has been registered by NBAGR in 2019 (Accession Number: INDIAN_DONKEY_0400_HALARI_05002). The population of Halari donkeys has fallen from 1,112 in 2015 to 662 by 2020 (https://ruralindiaonline.org/en/articles/7000-a-litre-milking-donkey-breeders-dreams/) and is further declining, therefore, these donkeys may be recognized as a threatened breed in near future. It is well known for heat tolerance, disease resistance and low input system in comparison to regular donkeys as reported by Pal *et al*., (2020). The evolutionary studies on equines suggested prominent information on their origin, evolution and domestication (Renaud *et al*., 2018) but the data regarding donkeys have not been documented properly since there is a theory circulating that donkeys are believed to be domesticated twice (Schubert *et al.,*2014; Librado *et al.,* 2017; Rossel *et al.,* 2008; Beja Pereira *et al.,*2004). In India, scanty reports are available about characterization of equine germplasm (Gupta *et al.,* 2012; 2014; 2015; 2018; Pal *et al.*,

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2013, 2020, 2021) and it needs to be explored in depth. Few reports are also available on Halari donkey milk (Bhardwaj *et al.,* 2019; 2020). Halari donkey, being the newly introduced breed (Pal *et al.,* 2020), possesses the possibility of understanding the genetic characterization and their diversity with reference to the complete mitochondrial genome of *Equus asinus*.

Molecular markers are well known for their significance in determining genetic diversity and maternal lineage distribution especially mitochondrial DNA since they are studied to be evolving much faster than nuclear DNA (Brown *et al*., 1993; Gupta *et al*., 2015, Gupta *et al*., 2018). The mitochondrial DNA is of around 16,569 bp with 37 genes being in a circular shape. It comprises of D-loop variable region that plays a major role in understanding the maternal genetic signatures among living beings and the cytochrome-b gene. The D-loop is a non-coding region with a high mutation rate (Slatkin, 1994). This helps in the identification of closely related species of animals (Brown *et al*., 1996; Gupta *et al*., 2015). The information about the genetic structure and variations in Halari donkeys is still rare owing us to design this study.

## MATERIAL AND METHODS

### Sample collection and DNA isolation

For the present study, whole blood from 23 random samples designated as (HL-1 TO HL23) belonging to the Halari donkey was collected from its breeding tracts in Saurashtra region of Gujarat, India, aseptically following the guidelines of IAEC and approval from the National Research Centre on Equines. Genomic DNA extraction was done from these whole blood samples using the DNA isolation kit following the recommended protocols by Promega (catalogue no. A5081). The quality of DNA was checked in 1% Agarose gel electrophoresis and Qubit 3.0.

### Polymerase chain reaction

The isolated DNA was subjected to amplification with a 50 µl volume of the reaction mixture of D-loop that contains 10x reaction buffer, 1.5 mM $MgCl_2$, 0.2 µm of forward and reverse primer, 0.2 mM of dNTPs, Taq Polymerase enzyme (Thermo Fisher Scientific) and 2 µl of working genomic DNA. The reaction cycle involved initial denaturation at 94°C for 4 minutes for 35 cycles followed by 94°C for 30 seconds of denaturation, 53°C for 45 seconds of annealing temperature, 72°C for 45 seconds of extension, 72°C for 10 min of final extension. The obtained products were checked on 1.8% Agarose gel to confirm the amplification of genes with the DNA.

The primers as reported by (Han *et al*., 2014); for the D-loop gene (542 bp) of mitochondrial DNA dlF 5′-TGAAACTATACCTGGCATCTGG-3′ and dlR 5′-CATGGACT GAATAACACCTTATGG-3′ were selected for the study.

### Data analysis

The obtained good quality sequences as determined by chromatograms of 23 Halari donkeys were aligned with the reference genome of mitochondria using the ClustalW of MEGA 7.0 (Smith *et al*., 2009) and were further subjected to the diversity analysis using DNASP V6 (Rozas *et al*., 2017). Phylogenetic analysis was carried out using MEGA (Smith *et al*., 2009) with 1000 bootstraps and Tajima Nei distance parameter (Tajima and Nei, 1993) using neighbour-joining phylogenetic analysis. A median-joining network has also been constructed using NETWORK 10.1 (Bland *et al*., 1999). A graph of mismatch distribution was drawn using NETWORK and genetic distances among the haplotypes using MEGA.

## RESULTS AND DISCUSSION

The nucleotide positions to the sequences were assigned based on the *Equus asinus* complete mitochondrial genome (NC_001788.1) from 15703 nts to 16245 nts making up to 542 bp of the D-loop gene. A total of 39 sites were found with 24 variable/polymorphic sites and 15 monomorphic/ invariable sites. A total of 8 haplotypes were detected and the genetic variation patterns were determined using this haplotype data. The haplotype distribution indicated that haplotype 3 is shared by the highest number of sequences (n=9) followed by haplotypes 5, 6 and 8 being shared by one sequence each. The haplotype 1 present in HL-4 is shared by the similar haplotype identified in the reference sequence. Such variations of D-loop sequences indicate the rapidly evolving and high mutation rate of the gene (Saccone *et al*., 1991) that can be due to gene flow and environmental changes (Hartl and Clark, 1997; Hirayama *et al*., 2010). The first report on Turkish donkey breeds was reported by Özkan Unal *et al*. (2020) and the study provided information of the genetic characterization in donkeys belonging to seven geographical locations of Turkey. This study revealed 54 haplotypes out of which 28 were found to be unique and 26 were shared among 16 different populations of Turkey donkeys with 315 donkeys. No proper clustering was observed in the median joining tree constructed. Another study involving 26 individuals belonging to 5 Chinese donkey breeds revealed 11 haplotypes. The study also suggested a possibility of reduction in the genetic diversity of Chinese donkeys recommending in developing better conservation techniques (Lei *et al*., 2005).

It has already been established that the modern donkeys have originated from Nubian and Somalian lineages (Kimura *et al*., 2011). To develop a better understanding of their lineages, 171 obtained sequences and 563 published sequences belonging to various regions including Asian, European, African samples were subjected to genetic diversity analysis with the help of D-loop by Ma *et al*. (2020). The results indicated a non-simultaneous domestication of these lineages that further influenced the human civilization accordingly.

A very recent study on Brazilian donkeys has been conducted with 30 donkeys belonging to three different Brazilian breeds in which 5 haplotypes have been detected (Alves *et al*., 2022). This study aimed in focusing on the

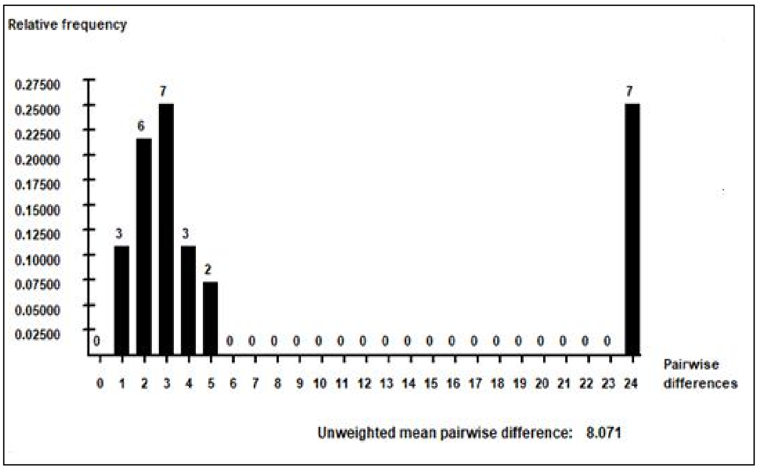

**Fig 1:** Mismatch distribution of the Halari donkey sequences.

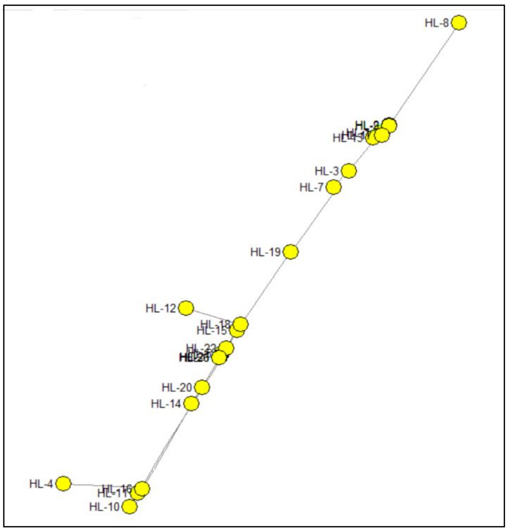

**Fig 2:** Median-joining network phylogeny of the sequences.

conservation of native Brazilian donkeys since their population has been drastically decreasing. This study reported the first ever work done on Brazilian donkeys and the results indicated high levels of genetic diversity and distance in the group. D-loop has always been a topic of interest due to its ability of identifying the maternal lineages and determining the genetic relationships among breeds using phylogenetic analysis. However, donkey's phylogeny has not been properly recorded. In a study conducted by Mazzatenta *et al*. (2021) D-loop of critically endangered breeds of Italian donkeys, Martina Franca, Ragusano, Pantesco and Catalonian has been evaluated. For a sample set of 77 individual DNA, 56 haplotypes were identified. The obtained data indicated the loss of variability within the taken breeds. The study also confirmed that the maternal lineages were well preserved in Marta Franca and Ragusano breeds. This further confirms the significance of D-loop and its application in studying the maternal inheritance of equines.

The haplotype diversity in the Halari donkey population was found to be 0.8152. The haplotype diversity increases with the increase of recombination and SNP frequency which results in an increase in genetic diversity (Stumpf, 2004). The results indicated that the Halari donkeys possess a nucleotide diversity of 0.12811 with a GC content of 0.422 which indicates a mild diversity among the animals. The average number of nucleotide differences was determined to be 4.99638. The genetic distance within the population was determined using the Kimura 2 parameter with an average of 1.4000. Graph 1 for mismatch distribution was drawn using NETWORK (Fig 1).

The mismatch distribution indicated the genetic differences between the pairs within the sample and peaks in the graph indicate the population growth. Although they indicate the population expansion, it doesn't affect the population structure among the samples (Harpending *et al*., 1994). We performed the Tajima's neutrality test to examine the population history of the individuals. The result (D= -2.039) indicated the positive selection and expansion of the population (Tajima, 1989). This can be due to the less frequency of polymorphisms. The trees (Fig 2 and 3) revealed a close relationship of one of the donkeys (HL-4) and grouped with the reference genome (NC_001788.1). However, they are not diversified enough to be classified into sub-populations. In order to determine the population structure of the Halari donkey demographical and hierarchical criteria need to be determined for a better management plan (McCracken *et al*., 2001). Median-joining network tree indicated close clustering of the samples.

In our study, we tried to establish the study regarding Halari donkeys on which a minimal amount of work has been done using the D-loop gene to identify the genetic relationships among the populations. The detection of 8 haplotypes in 23 donkeys indicates the existence of genetic diversity among the Halari donkey population. The study also determined high haplotype and nucleotide diversity values within the population. In summary, a high level of genetic diversity in Halari donkeys necessitates further breeding programs for its future development. This kind of high level of genetic diversity indicates a better scope in

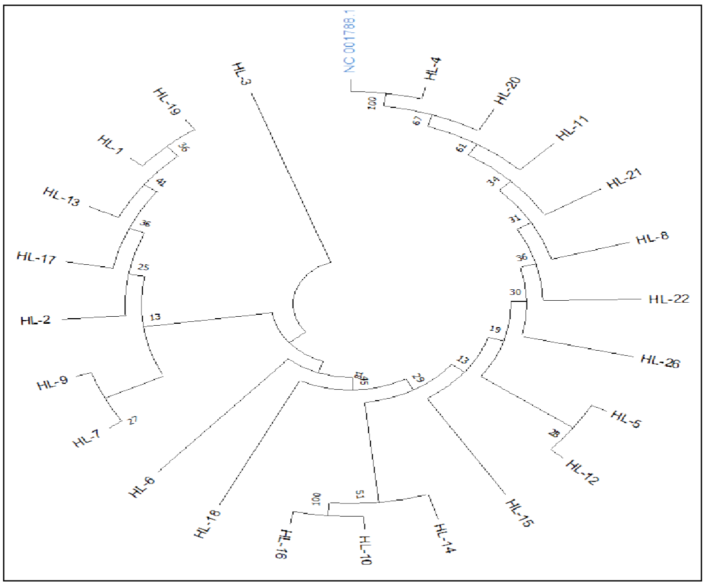

**Fig 3:** Neighbour-joining tree with 1000 bootstraps using Tajima Nei distance parameter.

understanding the evolution and conservation of Halari donkey breeds. Such conservation of genetic information helps in preserving endangered species as well as improving the productivity of livestock animals (Barker *et al*., 1994). It is necessary to work on the data more to clearly understand the distribution of clades and arrangement of the haplogroups.

## CONCLUSION

In conclusion, this study on the genetic characterization of mitochondrial D-loop gene of Halari donkey further contributes to the determination of its genetic diversity and its phylogenetic relationships and may be helpful in conservations, management and breeding of these native donkeys of India.

**Conflict of interest:** None.

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