DNA BARCODE OF MANIPUR PONY AND ITS GENETIC DIVERSITY BASED ON MITOCHONDRIAL D-LOOP

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Abstract:

Manipuri pony is a breed of horse and geographically distant from the other five recognized horse breeds found in the Indian subcontinent, viz., Marwari, Spiti, Zanskari, Bhutia and Kathiawari. There is high demand for this animal in various areas of the country as one of the most suitable pack pony in the hilly terrains. Today, the ponies are used as polo mounts, for racing, and in the military, where their speed, stamina, and intelligence are well-respected. However, the population of this unique animal is decreasing at an alarming rate. Government of India (2007) has pointed out the need of special conservation efforts on indigenous Manipuri Pony due to facing danger of extinction.

The Manipuri pony is referred and mentioned in most of the general literatures of equine and the breed is believed to be the descendant of Asian wild horse. However, no molecular study is available and as such the genetic relationship of this particular breed with the other Indian breeds as well as with the world wide breeds is unknown. It is apparent that development of species specific molecular marker, understanding the genetic diversity and evolutionary story is essential for the judicious exploitation and conservation of equine resources. In the context, DNA barcoding has been acclaimed for the ability to quickly cataloguing the biodiversity on the planet and to help determine where to focus for conservation efforts. Studying the D-loop region of the mitochondrial (mt) genome would help to understand the genetic diversification of domestic horses and for establishing the genetic relationships among the breeds.

The mitochondrial gene cytochrome c oxidase subunit I (COI) can serve as a uniform target gene for the development of DNA barcode in animal kingdom. However,
primer to amplify the barcode region is lacking in certain taxa and cross species Polymerase Chain Reaction (PCR) primers are used to amplify the targeted gene. In the present study, we validated a cross-species primer combination to generate DNA barcode sequence of Manipuri pony (*Equus caballus*). Different primers of common taxa were empirically tested *in silico* for their feasibility to generate the targeted fragment of *COI* gene. Based on the critical criterion of DNA barcoding, forward primer for bird DNA barcoding (Bird AF) and reverse primer for fish DNA barcoding (Fish AR) were selected as cross primer pair to amplify Manipuri pony's partial *COI* gene, which give an *in silico* PCR product of 699bp. This has also been verified by *in vitro* PCR amplification using genomic DNA extracted from hair samples of Manipuri pony. The sequence similarity and homology search in NCBI GenBank nucleotide database using BLASTN algorithm confirmed the result. Thus, the use of cross-species primers successfully resulted the development of agreed upon barcode fragment of the native horse of Manipur, and the first DNA barcode of Manipuri pony was submitted to NCBI vide accession no. JN228963.

The analysis was done on the forty *COI* sequences which include seven extant species of Equidae. The pairwise genetic distance among the species of Equidae ranged from 0.5% to 9.6% with an average of 5.5%. Conspecific individuals of this family showed the sequence divergence in the range of 0.2 % to 0.7% with a mean of 0.5%. The minimum congeneric distance was reflected between *Equus hemionus* and *Equus kiang* being 0.2%.

The mt D-loop region which is the most variable part of the mt genome has been importantly used for understanding the genetic diversification of domestic horses and for establishing the genetic relationships among breeds. Primer to amplify the D-loop region of Manipuri pony was designed by using different software. Studying on the 247 bp region of mt D-loop region was carried out to estimate the genetic diversity and to understand the maternal lineages of Manipuri pony. The dataset comprised of eleven numbers of self-developed sequences of Manipuri pony, fifty-nine and thirty-five number of database sequences of Indian horse breeds and other worldwide breeds respectively. A total of thirty-five haplotypes was identified with a high level of genetic diversity in the Indian
breeds. Marwari showed the highest genetic diversity (0.985) and Zanskari the least (0.864). A total of seven major mtDNA haplogroups (A-G) was identified in the Indian horse breeds that indicated the abundance of mtDNA diversity and multiple origins of maternal lineages in them. The majority of the studied sequences of Indian breeds (33.3%) were grouped into haplogroup D and least (3.9%) in haplogroup E. The Manipuri breed showed the least $F_{ST}$ distance (0.03866) with Marwari among the Indian breeds and with Thoroughbred horse among the worldwide. This study indicated a close association between Manipuri pony and Thoroughbred.

The AMOVA analysis also revealed the distinct population of Zanskari among other Indian horse breeds. Population pairwise differences ($F_{ST}$) further confirmed the breed differentiation. Calculating genetic differentiation showed closest affinity of Manipuri with Marwari among Indians and with Thoroughbred from broad geographical regions.

Our result shows a relatively high genotypic diversity within and between the analysed horse populations. The genetic diversity information based on mtDNA typing is important baseline data for the future breed conservation strategy. The high diversity of the mt D-loop region within the studied horse breeds confirms a differentiated ancestry, previously indicated by several authors.

The phylogenetic analyses conducted on COI and D-loop data sets separately lead to different results. Phylogenetic tree based on COI sequences identified two monophyletic groups: caballines and non caballines which D-loop cannot confer. The analysis showed the close grouping of individuals of a single species, regardless of where they were collected and showed shallow intraspecific and deep interspecific divergences. But there is blending for *Equus hemionus* and *Equus kiang* which they also shared a 28 bp deletion on the D-loop sequences.

**Keywords:** Manipur pony (*Equus caballus*), mitochondrial genome, cytochrome c oxidase subunit I (COI), cross species primer, *in silico*, sequence divergence, D-loop, haplogroup, genetic diversity, maternal origin.