

MITOCHONDRIAL CYTOCHROME-B AND D-LOOP SEQUENCE BASED GENETIC DIVERSITY IN MAREECHA AND BAREELA CAMEL BREEDS OF PAKISTAN

M. E. Babar¹, T. Hussain*¹, A. Wajid², A. Nawaz³, A. Nadeem², S. A. Shah², M. A. Shahid⁴, N. Ahmad⁵, K. Javed⁵ and M. Abdullah⁵

¹Department of Molecular Biology, Virtual University of Pakistan, Lahore; ²Institute of Biochemistry and Biotechnology, ⁵Department of Livestock Production University of Veterinary and Animal Sciences Lahore, 54000, Pakistan; ³Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Balochistan, Pakistan; ⁴Camel Breeding and Research Station, Rakh Mahni, District Bhakkar, Punjab, Pakistan.

First two authors contributed equally

*Corresponding author: tanveer.hussain@vu.edu.pk

ABSTRACT

This is the first study designed on mitochondrial Cytochrome b gene (*Cyt-b*) and Displacement (D) loop sequence diversity in two camel breeds (Mareecha and Bareela) of Pakistan to assess the status of genetic diversity and differentiation. Genomic DNA was extracted from total of 48 samples (Mareecha = 25 and Bareela = 23). The complete *Cyto-b* gene sequences (1140bp) analysis showed four parsimony informative sites were at position 137, 146, 337 and 806 and five haplotypes were constructed, indicating low *Cyto-b* gene genetic variability in selected two Pakistani camel breeds. Analysis of complete mt-DNA D-loop (1214 bp) sequences revealed eight different haplotypes resulting from 32 polymorphic sites (2.63% of all sites), of which 31 sites were singleton variable sites and one site was parsimony informative polymorphic site. The phylogenetic analysis showed two clades of camel (*Camelus*), dromedary and Bactrian populations that came out as distinct lineage and showed distinct genetic distance between them, and all Pakistani camel haplotypes were clustered with dromedary camels confirmed their genetic architecture as dromedary camels. Genetic variability assessment on camels is important to preserve this genetic resource and to develop future breeding plans to improve camel productivity.

Key word: *Cytochrome b* and *D-loop*, *Phylogenetic analysis*, *Camel breeds*, *Pakistan*.

INTRODUCTION

Presently, about 24.1 million camels live on this planet (FAO, 2012). Their family, called *Camiladae*, has two genera *Camalus* and *Lama*. Both genera share long necks, high water efficiency and two toes with padded feet. This pseudoruminant has some unique features as: horns/antlers are missing, hump (energy reservoir in the form of fats) is present, it walks on pads and what makes it very unique is its ability to conserve body water (Al-Swailem *et al.*, 2007). Humankind has been using camel for leather, fiber, hair, milk, meat, transportation, as a war, entertainment and sacrificial animal since 3000 BC (Vijh *et al.*, 2007). Their common habitats around the globe are arid, semi arid and desert areas. That is why Australian outback, Iran, India, Pakistan, Somalia, Sudan, Ethiopia and Saudi-Arabian Peninsula are the major habitat of camel population (Al-Swailem *et al.*, 2007). Pakistan is having 1 million camels being 2nd largest population in the world (Anonymous, 2012). Camel, as source of milk and meat, is getting attention of researchers. Its meat does not induce that much cholesterol and fat compared with other beefs. Meanwhile, camel milk has proved a significant alternate of protein to the people who are allergic to bovine milk.

Moreover, camel milk is seems to having vital potential for production of the recombinant proteins (Kaske *et al.*, 2001). Mitochondrial *Cyt-b* gene and D-loop region are most powerful markers for the characterization of different genetic resources (Goldstein & Pollock, 1997). Thus, the aim of this study was to discover the variation in the said regions of camel breeds of Pakistan. Mutations in mitochondrial genome are very rapid and distinctive among species, but when it comes to within species i.e. breeds these are very rare. Their rarity helps us to define breeds clearly. Side advantage of using *Cyt-b* gene and D-loop region is implications in defining milk production marker along with development of forensic analysis in case of parentage conflicts. The present study will set a platform for researcher to go further for marker assisted selection, better breeding and recombinant protein production in Pakistani camel (*Camelus dromedarius*) as it is important fraction of the animal genetic resource of Pakistan.

MATERIALS AND METHODS

Sample collection and DNA extraction: A total of 48 samples were collected from Mareecha (n=25) and Bareela (n=23) camel from Camel Breeding & Research

Station (Bhakkar), Rakh Mahni, Mithra, Bahawalpur and different districts of Baluchistan. Total genomic DNA was extracted from blood using standard procedure devised by Sambrook and Russel (2001).

PCR Amplification and sequencing: Four pairs of primers were designed to amplified the complete sequence of mitochondrial *Cyt-b* gene and D-loop from the sequence available in NCBI (Accession no JN632608) using the Primer3 software v. 0.4.0 (Rozen and Skaletsky, 2000) (Table 1). PCR amplifications were carried out in 25 μ L reaction mixtures containing 50 ng of DNA template, 10 pmol of each primer, 2.5 mM MgCl₂, 100 μ M of dNTPs mix and 1.5 U of Taq DNA polymerase (Fermentas, Thermo Fisher Scientific Inc. USA). PCR was carried out in BioRad thermocycler using initial denaturation of 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30s and extension at 72 °C for 30s, followed by final extension at 72 °C for 7 min. The PCR products were purified and sequenced on an ABI 3130XL genetic analyzer (Applied Biosystems, Foster, CA, USA) using a BigDye Terminator cycle sequencing kit (Applied Biosystems).

Table 1. Primer sequences of camel mitochondrial Cytochrome b gene and D-loop

5'– 3'	Primer sequence
Cyto 1F	TTCATTGACCTCCCTGCTC
Cyto 1R	AGGTTGGTGATGACTGTTGC
Cyto 2F	GGATACGTAAGTCCATGAGG
Cyto 2R	TTGCTGGGGTGTAGTTGTCT
Dloop1F	AAAATCCCATTCACCCCTA
Dloop1R	ATGCTGGCCGTTGGTATTAG
Dloop2F	TTGCTGGGGTGTAGTTGTCT
Dloop2R	TTGCTGGGGTGTAGTTGTCT

Analysis of sequences data: The sequences of *Cyt-b* gene and D-loop were aligned against reference sequence by using BioEdit 7.0.9.0 (Hall, 1999). Sequence results were cleaned and SNPs were identified with the help of Multiple Sequence Alignment (MSA) strategy using ClustalW software package (Thompson *et al.*, 1997). DnaSP software (Librado and Rozas, 2009) was used to find polymorphic sites, number of haplotypes, haplotype and nucleotide diversity. Using the computer program MEGA V5.05, a Neighbor-Joining (NJ) phylogenetic tree based on two parameter models were reconstructed to carry out phylogenetic analysis. (Tamura *et al.*, 2011).

RESULTS AND DISCUSSION

Sequence analysis of Camel Cytochrome b gene and D-loop: The complete mitochondrial *Cyt-b* gene and D-loop sequences were detected for 48 Pakistani camels, 25

for Mareecha and 23 for Bareela. The analysis of *Cyt-b* gene revealed five haplotypes resulting from four parsimony informative sites at position 137, 146, 337 and 806, of which one was synonymous and three were Messense mutations. We found three haplotypes in Mareecha and two haplotypes in Bareela. Analysis of the variable sites revealed two transversions (T>A & T>G) and two transitions (T>C & A>G) (Table 2), and no insertions/deletions were observed. The averaged nucleotide frequencies of T, A, C and G were 26.1, 31.7, 29.1 and 13.1%, respectively. The average haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.8333 \pm 0.1220 and 0.00187 \pm 0.00040 respectively, indicating abundant genetic diversity in Pakistani camel breeds.

Table 2. Polymorphic sites detected in Cytochrome b gene of Pakistani camel breeds

Position	Wild type	Mutant type	Transition/Transversion
137	T	C	Transition
146	T	A	Transversion
337	T	G	Transversion
806	A	G	Transition

Mitochondrial D-loop, also known as mitochondrial control region, is a non-coding region. It is a superlative molecular marker for the study of genetic variation between and within closely related species due to more polymorphic as compare to other mitochondrial regions and its base substitution rate is 2.8 to 5 times of other sections of mt-DNA (Xu *et al.*, 2010). We also sequenced complete mitochondrial D-loop (1214 bp) of both breeds. A total of 32 variable sites (2.63% of all sites) were observed, of which 31 sites were two variants singleton variable sites 110, 190, 429, 677, 678, 679, 683, 684, 699, 700, 704, 717, 719, 720, 721, 722, 725, 726, 727, 728, 731, 732, 734, 739, 740, 741, 742, 775, 876, 939, 941 and one site was parsimony informative polymorphic site 460. We found a total of eight haplotypes, four in each breed. They did not share any haplotypes but a total of nine mutations were shared by the two subpopulations. The average haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.8940 \pm 0.0110 and 0.01008 \pm 0.00138, respectively.

The Phylogenetic tree constructed for mt *Cyt-b* gene:

The NJ (Neighbor-joining) tree was constructed using MEGA v5 program with bootstrap value 1000. The phylogenetic analysis showed two clades of camel (*Camelus*), dromedary and Bactrian populations that came out as distinct lineage and showed large genetic distance between them (Figure 1). The clade A contained all dromedary camels/ single humped camels with all identified Pakistani haplotypes, and clade B contains Bactrian camels or two humped camels. The phylogenetic

tree showed that sheep (*Ovis aries*) is comparatively closer to camel (*Camelus*) as compare to other species.

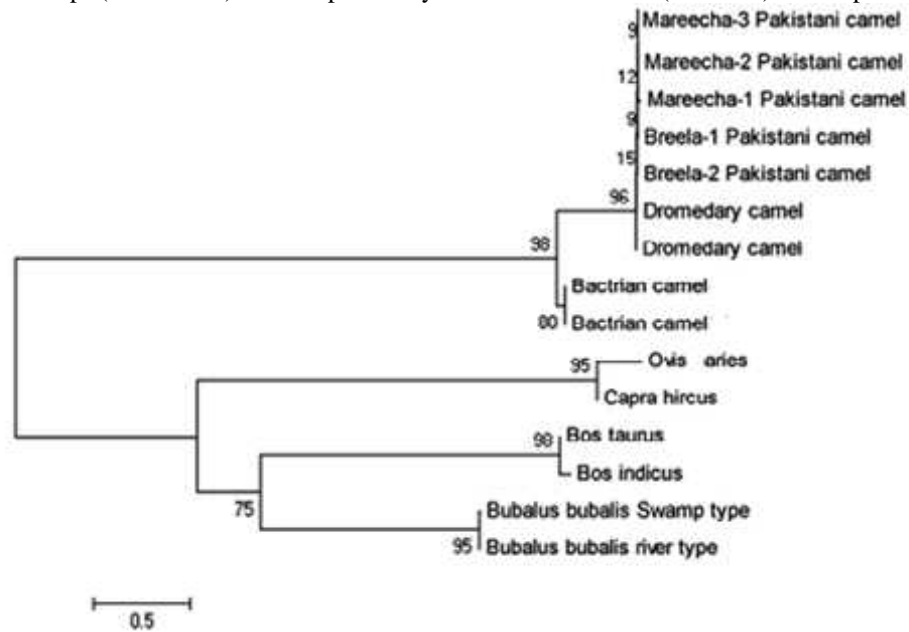


Fig. 1: Neighbor-Joining Phylogenetic tree of Pakistani Mareecha and Bareela breeds with already reported Dromedary Bactrian camel, *Ovis aries*, *Capra hircus*, *Bos taurus*, *Bos indicus*, *Bubalus bubalis* (river and swamp type) using bootstrap value 1000.

The Phylogenetic three constructed for mtD-loop: In this study, phylogenetic analysis based on complete mtD-loop sequences (1214bp), showed four Pakistani camel haplotypes from both breeds that were found in the same clade with already reported dromedary camels (Figure 2). Like phylogenetic tree constructed for *Cyt-b*, the wild Bactrian and Bactrian camel separated as another cluster.

Ovis aries seems closer to camels as compared to other species in the tree, *Bos taurus*, *Bos indicus*, *Bubalus bubalis* (river and swamp type) maintained their own biological positions. The phylogenetic tree constructed for mtD-loop showed very similar result with *Cyt-b* gene tree.

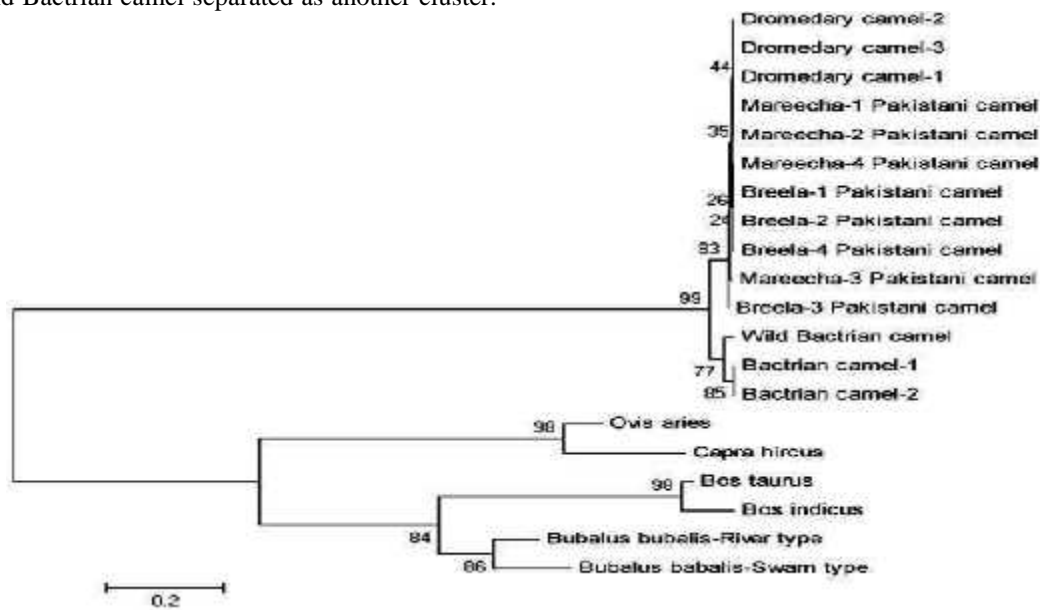


Fig. 2: Neighbor-Joining Phylogenetic tree of Pakistani Mareecha and Bareela breeds with already reported Dromedary, wild Bactrian and Bactrian camel, *Ovis aries*, *Capra hircus*, *Bos taurus*, *Bos indicus*, *Bubalus bubalis* (river and swamp type) using bootstrap value 1000.

It is concluded that complete mitochondrial *Cyt-b* gene and D-loop sequence analysis in Mareecha and Bareela breed confirmed its genetic makeup as dromedary camels that are closer to other reported dromedary camels and they are well distanced from Bactrian camels however *Ovis aries* showed higher similarity as compared to *Capra hircus*, *Bos taurus*, *Bos indicus*, *Bubalus bubalis*. Ahmed *et al.*, (2013) studied the mitochondrial genes in Arabian camel and reported that *Cyt-b* in mitochondrial membrane attained the largely substitutions of amino acids. Molecular characterization by using microsatellite markers (Parikh *et al.*, 2012; Sushma *et al.*, 2014), mitochondrial and nuclear markers (Chuluunbat *et al.*, 2014) of different camel breeds were carried out for genetic diversity analysis. This is the first report on camel genetic diversity from Pakistan however more studies with increased number of samples including other camel breeds of Pakistan is recommended for getting more insight. Genetic variability assessment on camels is important to preserve this genetic resource and to develop future breeding plans to improve camel productivity, and also the future research activities with larger sample size and other molecular markers such as microsatellites and Y chromosomal data could provides another views on their phylogenetic architecture.

REFERENCES

- Ahmed, M. M., S. A. El-Shazly, S. M. Sayed, , S. A. M. Amer (2013). Molecular study of energy related mitochondrial genes in Arabian and Bactrian camels. *American J. Bioch. and Biotech.*,9(1):61
- Al-Swailem, A. M., K. A. Al-Busadah, M. M. Shehata, I. O. Al-Anazi and E. Askari (2007): Classification of Saudi Arabian camel (*Camelus dromedarius*) subtypes based on RAPD technique. *J. Food, Agri. and Env.* 5 (1): 143-148.
- Anonymous. (2011-12). Pakistan Economic Survey. Government of Pakistan, Finance division, Economic Advisor's Wing Islamabad.
- Chuluunbat, B., P. Charruau, K. Silbermayr, T. Khorloojav and P. A. Burger (2014). Genetic diversity and population structure of Mongolian domestic Bactrian camels (*Camelus bactrianus*). *Animal Genetics*. doi: 10.1111/age.12158
- FAO. (2012). Domestic Animal Diversity Information System. (www.fao.org/DAD-IS). Web Page.
- Goldstein, D. B and D. D. Pollock (1997). Launching microsatellites: a review of mutation processes and methods of phylogenetic interference. *J. Hered.* 88(5): 335-342.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid S.* 41: 95-98.
- Kaske, M., B. Elmahdi, W. von Engelhardt and H.P Sallmann (2001). Insulin responsiveness of sheep, ponies, miniature pigs, and camels: results of hyperinsulinemic clamps using porcine insulin. *J. Comp. Physiol. B.* 171: 549-556.
- Librado, P. and J. Rozas (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451-1452.
- Parikh R.C., N.A. Patel, Y.D. Padheriya, K.N. Wadhvani, D.N. Rank (2012). Molecular characterisation of kachchi camel (*Camelus dromedarius*) using microsatellite markers. *J. Camel Practice and Research.* 19(2):153-157.
- Sambrook J. and D. W. Russell. (2001). Preparation and analysis of eukaryotic genomic DNA. In: *Molecular Cloning: A Laboratory Manual* (Ed. by J. Sambrook & D.W. Russell), pp. 6.11-6.14. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sushma P., Sharique, A. A., Priyanka B., Jyoti J., Upasna S. and R.K. Vijh (2014). Genetic Characterization of Malvi camel using Microsatellite markers. *DHR Intl. J. Biomedical and Life Sciences* 5(1): 286-296
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar (2011). *Mol. Bio. Evol.* 28: 2731-2739.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Bio. and Evol.* 28: 2731-2739.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins (1997). The Clustal-X windows interface: flexible strategies for multiple sequences alignment aided by quality analysis tools. *Nucl. Acids. Res.* 25: 4876-4882.
- Vijh, R. K., M. S. Tania, B. Mishra and S. T. B. Kumar (2007). Genetic diversity and differentiation of dromedarian camel of India. *Anim. Biotech.* 18 (2): 81-90.
- Xu, X., X. Wu and Z. Yu (2010). The mitogenome of *Paphia euglypta* (Bivalvia: Veneridae) and comparative mitogenomic analyses of three venerids. *Genome.* 53(12):1041-1052.