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MOLECULAR DIVERSITY AND PHYLOGENETIC ANALYSIS OF EIGHT DROMEDARY CAMEL BREEDS OF PAKISTAN BASED ON MITOCHONDRIAL *ATP6* AND *ATP8* GENES

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This work was designed to detect genetic polymorphism in mitochondrial *ATP6* and *ATP8* genes in eight Pakistani camel breeds (*Camelus dromedarius*) including Mareecha, Barela, Kachi, Kharani, Thari, Pahari, Watni and Mix-bred (non-descriptive). The genetic relationships among the selected breeds belonging to miscellaneous geographical regions were also carried out through sequences of selected genes. A total of 842 bp including full CDS of both *ATP6* and *ATP8* genes showed 11 polymorphic sites in all the selected camel breeds. Five polymorphic sites were single variable and six were found parsimony informative sites. The neighbor joining phylogenetic tree was constructed through MEGA 6 program. All Pakistani camel breeds were confirmed as *dromedaries*. The phylogenetic tree with 23 other mammalian species reconfirmed the classical biological classification. To the best of our knowledge this is the earliest report on selected genes in Pakistani dromedary camel breeds.

Keywords: Dromedary camel, *ATP6* and *ATP8* genes, polymorphisms, phylogenetics.

INTRODUCTION

Camel (*Camelus dromedaries*) is an animal of immense economic value, providing useful products and services e.g. milk, wool, meat and drought power to mankind. Camels have high forbearance ability adjacent to a very harsh, severe and dehydration-prone environment (Schwartz, 1992; Yaqoob and Nawaz, 2007). Camel has rare physiological characteristics; their body temperatures may fluctuate from 34 to 42.1°C during the whole day, they may tolerate loss of water more than 32% and their ability to store more than 100 liter of drinking water in their body (Jasra and Mirza, 2005). Due to these qualities, they have a unique ability to adapt the desert lifestyle.

Pakistani camel is of two types, riverine and mountain/hilly. Now, there are 21 documented breeds of camels in Pakistan. Camels of seven breeds are present in Balochistan province in of Pakistan i.e. Brahvi, Kachhi, Kharani, Lassi, Makrani, Pishin, Rodbari, whereas, in Khyber Pakhtunkhwa, four breeds i.e. Gaddi, Ghulmani, Khader and Maya, in Punjab, there are five breeds i.e. Bagri, Barela or Thalocha, Cambelpuri, Kala-Chitta and Mareecha and four breeds are documented in Sindh province of Pakistan i.e. Dhatti, Khari, Larri or Sindhi and Sakrai. Out of these, Mareecha, Dhatti, Larri, Sakrai are riverine type while Kohi, Cambelpuri are mountain/hilly sort of animals (Heston *et al.*, 1985; Isani and Baloch, 2000)

The mitochondrial (mt) DNA can be used as a best marker to study the phylogeny almost in all mammalian species, due to its mutation rate as compared to nuclear DNA (Avise, 1994; Stanley *et al.*, 1994). The mtDNA becomes most appropriate target for some of genetic studies (Galtier *et al.*, 2009) The mitochondrial genome of Bactrian camel has 16,680 bp (Cui *et al.*, 2007). Selected mt *ATP6* and *ATP8* genes are involved in energy production in unicellular and multi-cellular organisms. The mammalian mitochondrion consists of 16 subunits through which mt*ATP6* and *ATP8* genes are essential. Research on mutations in mitochondrial genes explains the evolution process in detail (Di Rocco *et al.*, 2009). In the current study, mitochondrial genes i.e. *ATP6* and *ATP8* were sequenced for understanding the evolutionary relationship of eight camel breeds found in Pakistan. Conducting research on the camel genome will help to identify genes of great importance, that will make clear that how camels has adjusted themselves in their harsh habitat (Schwartz, 1992; Al-Swailem *et al.*, 2007).

MATERIALS AND METHODS

***Sample collection and DNA extraction*:** Blood samples from 79 true representative animals from the eight different camel breeds (Barela, Mareecha, Kachi, Kharani, Pahari, Thari, Watni and Mix-bred) were collected from various government livestock farms and from their respective home tracts in different regions of the country. The DNA was extracted by inorganic method of extraction as described by Sambrook *et al.* (1989) and Hussain *et al.* (2015). Extracted DNA samples were quantified through NanoDrop 2000/2000c (Thermo Scientific USA).

***Primer designing*:** Specific primers were designed from NCBI, GenBank sequence database (www.ncbi.nlm.nih.gov) given in Table 1. The camel sequence (accession Number: JN632608, *Camelus dromedarius* isolate Morocco mitochondrion, complete genome) was used as reference for this study. The size of *ATP8* gene is 204 bp followed by *ATP6* gene of 681 bp. Both the genes are continuous with overlapping region of 43 bp. Primers were designed by Primer fox software (www.primerfox.com). The total product size of the primer pair was 890 bp (Table 2)

Table 1. Length of mitochondrial *ATP 6* and *ATP 8* genes with overlapping region.

Region	Length (bp)
ATP synthase subunit 8 gene	204bp
ATP synthase subunit 6 gene	681bp
Overlapping region	43bp

Table 2. Mitochondrial *ATP8* and *ATP6* Genes Primer Pair for Pakistani dromedary camel.

Primers	5' to 3' Sequence	Product size
CdATP8/6-F	AGCCATGACCCCTCCTTAGT	890 bp
CdATP8/6-R	TGGTATGCGTGAGTCTGGTG	

***Amplification, sequencing and software analysis*:** Primers were found best at annealing temperature of 54°C using standard PCR protocol using final reaction mixture of 25 µL. PCR products were run on 1.2% gel electrophoresis and positive samples were precipitated using absolute ethanol before sequencing using ABI Genetic Analyzer 3130 xL (San Diego California) through Sanger's sequencing method.

All the sequences were aligned with the help of online software blast2 sequence (http/www.ncbi.nlm.nih.com) and the sequences analysis and polymorphism identification were detected using CodonCode Aligner. The Neighbor Joining phylogenetic trees were constructed from the final consensus sequences with help of MEGA 6 program package (Tamura *et al.*, 2013). The neighbour joining phylogenetic tree was constructed among eight studied breeds of camel on the basis of consensus sequences of *ATP6* and *ATP8* genes from each breed. Support for individual branch of phylogenetic tree was assessed by Bootstrap percentages computed after 1000 replicates. The Pakistani breeds were compared with dromedary, domestic and wild Bactrian camel sequences from other parts of the world along with sequences of different mammalian species already reported on GenBank, NCBI (Table 3). DnaSP software package was used to identify the haplotype and nucleotide diversities and analysis of nucleotide polymorphisms (Librado and Rozas, 2009)

Table 3. *ATP6* and *ATP8* sequences of different Mammalian species obtained from GenBank, NCBI and used for phylogenetic analysis with dromedary camel breeds of Pakistan.

Animal	Species	GenBank Accession No.
Arabian Camel	*Camelus dromedaries*	JN632608
Bactrian Camel	*Camelus bactrianus*	AP003423
Wild Bactrian Camel	*Camelus bactrianus ferus*	EF507800
Guanaco	*Lama guanicoe*	EU681954
Vicugna	*Vicugna icugna*	FJ45689
Llama	*Lama glama*	AP003426
Alpaca	*Lama pacos*	NC002504
Cattle (humped)	*Bos indicus*	JN817303
Cattle (non-humped)	*Bos taurus*	HQ025805
Domestic yak	*Bos grunniens*	GQ464260
American bison	*Bison bison*	GU946998
Water Buffalo	*Bubalus bubalis*	NC006295
Domestic goat	*Capra hircus*	NC005044
Sheep	*Ovis aries*	NC001941
Peruvian guemal	*Hippocamelus antisensis*	JN632646
Gray brocket	*Mazama gouazoupira*	JN632658
Red brocket	*Mazama Americana*	JN632657
White tailed deer	*Odocoileus virginianus*	JN632672
Barasingha	*Rucervus duvaucelii*	JN632696
Mesopotamian fallow deer	*Dama mesopotamica*	JN632630
Yarkland deer	*Cervus elaphus yarkandensis*	GU457435
Formosan Sika deer	*Cervus Nippon taiouanus*	EF058308
Human	*Homo sapiens*	JF896800

RESULTS

***Genetic variations*:** After sequencing and alignment a total of 842 bp region of both *ATP6* and *ATP8* genes in all eight camel breeds were obtained. No deletion/insertion mutations were found. A total 29 mutations were detected on 11 different polymorphic sites. Five sites were singleton variable sites at position 153, 202, 249, 374, and 572, while six sites 216, 290,

447, 593, 779 and 785 were found parsimony informative sites. A total of nine haplotypes were generated from all sequences in eight camel breeds. The haplotype (Hd) and nucleotide diversity (Pi) were 0.5290 ± 0.1010 and 0.00181 ± 0.00047 respectively. Because all sequences obtained from different geographical regions across the country, the Tajima's D value -1.33564 designated signature of population's expansion.

The average number of nucleotide difference in all studied camel breeds were K: 1.52605. Haplotype 1 included Thari, Kharani and Pahari. Haplotype 2 included Kachi. Haplotype 3 was shared by three camel breeds Watni, Mix-bred and Mareecha. Haplotype 4 has a single breed Barela, Haplotpe 5 included Barela, Haplotype 6 have a single Mix-bred, Haplotype 7 included Mix-bred, Haplotype 8 included Mareecha camel and Haplotype 9 also included Mareecha camel breed.

Mareecha and Mix-bred represent three haplotyes (Hap3, Hap8, Hap9 and Hap3, Hap6, Hap7 respectively), while Barela showed two haplotypes (Hap4 and Hap5) and the remaining five camel breeds Pahari (Hap1), Kachi (Hap2), Thari (Hap1), Watni (Hap3) and Kharani (Hap1) revealed one haplotype. Haplotype1 is shared by three camel breeds including Thari, Kharani and Pahari while Haplotype3 is shared by Watni, Mix-bred and Mareecha breed.

***Phylogenetic analysis*:** The phylogenetic results showed the genetic relatedness of these breeds as the Mareecha and Pahari camel breeds of the Punjab are grouped together. Similarly, the camel breeds sampled from Balochistan province of Pakistan are also grouped close to each other i.e. Thari, Watni, Kharani and Kachhi (Fig. 1).

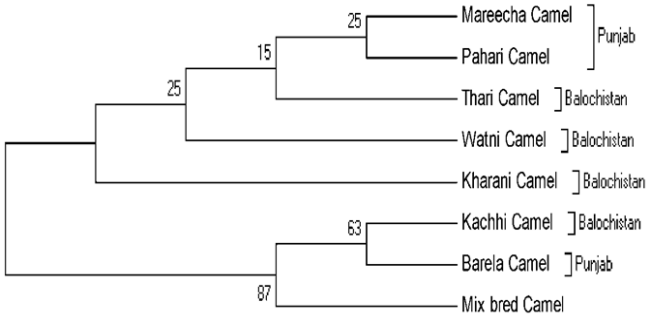

Figure 1. Phylogenetic tree of Pakistani Camel breeds (Province wise) based on *ATP6* and *ATP8* gene sequences using MEGA 6.1 (Neighbor-Joining Tree) using 1000 bootstrap value (rectangular view).

The *ATP8* and *ATP6* genes sequence data of studied breeds was compared with reported sequence data (available in GenBank NCBI). The Sheep (*Ovis aries*) *ATP8* and *ATP6* sequence was selected as outer group. All studied single humped camel of Pakistan (*Camelus dromedarius*) grouped together along with Arabian camel (*Camelus dromedarius*) sequence. The two humped camels (*Camelus bactrianus*) were also grouped together in another clade, confirming the genetic differences between two types of camel (Fig. 2).

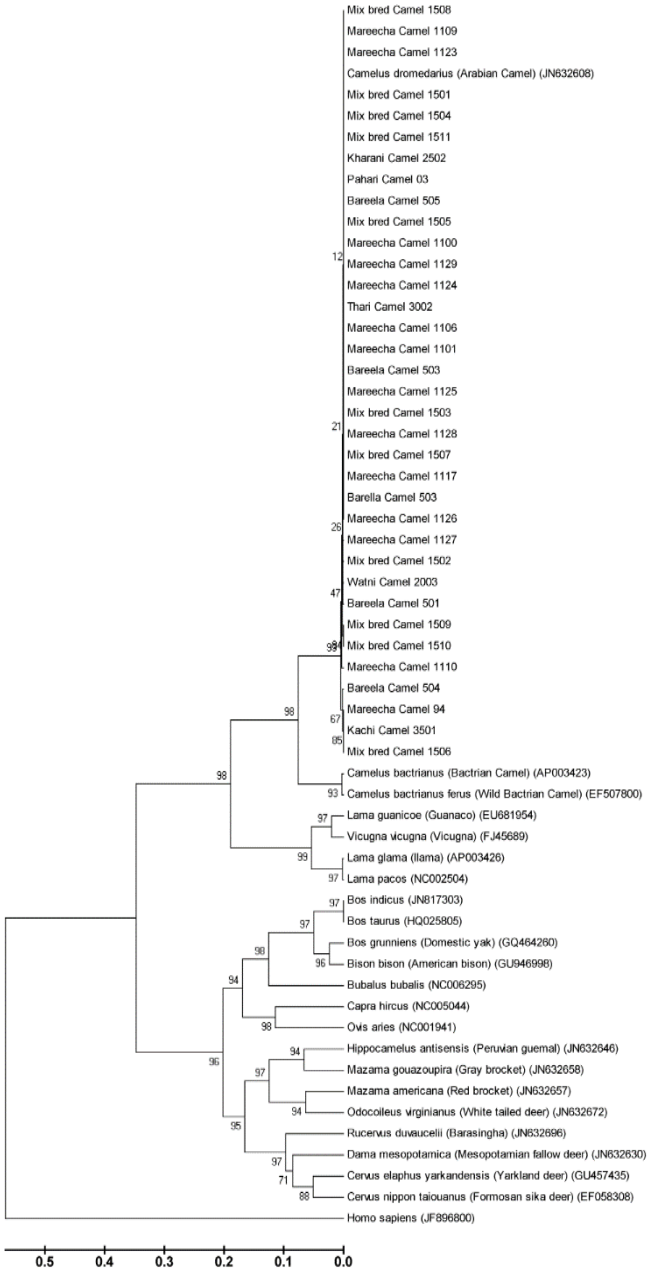

Figure 2. Neighbor Joining tree of selected camel breeds of Pakistan with other reported sequences from different mammalian species, constructed using MEGA 6.1.

DISCUSSION

The phylogenetic tree based on consensus sequences of eight Pakistani camel breeds gave an idea about their close genetic relationships. The tree was somehow following the geographical distances as the breeds from Balochistan and Punjab were grouped separately. Barela from Punjab and Kachhi from Balochistan showed closeness in the phylogeny they are found in close vicinity, therefore there might be chances of cross breeding between both breeds followed by the migration of local people from one area to other (Fig. 1).

There have been some studies on phylogeny of camel in the world based on mitochondrial genome, in which *Cyt b* and D-loop region of mitochondria were evaluated and phylogenetic relationships were also recognized among sub-species of South American Camelids, in which they included sequences of three of the four sub-species of *Lama guanicoe* (guanaco) and both of the *Vicugna vicugna* (vicuña) as well to check the relatedness, similar to the current study (Irwin *et al.*, 1991; Stanley *et al.*, 1994; Palma *et al.*, 2001). Another South American Camelid study, which validated the use of control region of mitochondrial genome as a molecular biomarker to deduce data on Camelid genetic relationships and population biodiversity (Mate *et al.*, 2004). Similarly, nucleotide sequence of a polyubiquitin gene (PUBC1) of (*Camelus dromedarius*) was also used to have an insight of genetic diversity in one of the Arabian camel study, which revealed that there are genetic variation, one of the 325 bp motif was 95 and 88% identical to human polyubiquitin genes B and C respectively and synonymous mutations were observed, while their amino acid sequence was 100% homologous (Al-Khedhairy, 2004). Microsatellite markers can also be served as a tool for determining population diversity, one of the Egyptian studies revealed that RAPD markers results showed genetic variation between and within different camel breeds. The phylogenetic relationship among the five camel breeds showed two groups. The first group includes Maghrabi, Baladi and Mowallad, while the second group of Sudani and Somali. This study showed that Mowallad breed was very close to both Baladi and Maghrabi which confirm the origin of Mowallad as a hybrid between Baladi and Maghrabi breeds (Mahrous *et al.*, 2011).

Another phylogenetic tree was constructed in the current study using haplotypes from Pakistani camel along with other species. The one clade in the phylogenetic tree was consisted of single humped camel (*C. dromedarius*) i.e. Pakistani and Arabian camel and two humped camel (*C. bactrianus*), while the next clade consisted of the biological cousins of camel i.e. Lama (*Lama glama*), Guanaco (*L. guanicoe*) and Vicugna (*Vicugna icugna*).

The second major branch of the phylogenetic tree consisted of all other mammals. This major branch was further divided into two sub branches i.e. Bovine family representing cattle i.e. *Bos taurus* (non-humped cattle) and *Bos indicus* (humped cattle) together, Yak (*Bos grunniens*) and American bison (*Bison bison*) together, then buffalo (*Bubalus bubalis*), the sheep (*Ovis aries*) and goat (*Capra hircus*) together. One of similar sort of Mongolian domestic bacterian camel study demonstrate that their local bacterian camel are in close relatedness to Chinese bactrian and dromedries (Chuluunbat *et al.*, 2014). One of the phylogenetic study of domestic and wild-type camels revealed that extant wild type two-humped camel may not share common ancestors with bactrian domestic camels is not the same sub-species in their maternal origins (Cui *et al.*, 2007; Ji *et al.*, 2009).

The second sub branch was consisting of other mammal species i.e. Peruvian guemal (*Hippocamelus antisensis*), Gray brocket (*Mazama gouazoupira*), Red brocket (*Mazama americana*), white tailed deer (*Odocoileus virginianus*) as they were grouped together. Similarly, the Barsingha (*Rucervus duvaucelii*), Mesopotamian fallow deer (*Dama mesopotamica*), Yarkland deer (*Cervus elaphus yarkandensis*) and Formosan Sika deer (*Cervus Nippon taiouanus*) were also grouped together (Fig. 2).

The *ATP6* and *ATP8* genes sequence of human (*Homo sapiens*) were taken as outer group and it clearly differed and separated from rest of the phylogenetic tree leaves i.e. camel, bovine, ovine, caprine and other mammals.

***Conclusion*:** Our results on the genetic architecture of Pakistani camel breeds produced base-line information on camel genetics. In general, the low genetic distances between the studied breeds can give an insight about their origin and evolution. This may clarify genetic relationships between and among different camel breeds. This study may be used to design proper breeding and conservation policies for camel in Pakistan to save this important genetic resource of the country.

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