The Journal of Animal & Plant Sciences, 26(2): 2016, Page: 437-444
ISSN: 1018-7081

COMPARATIVE ANALYSIS OF ATP6 MITOCHONDRIAL GENE DIVERSITY IN ARABIAN AND NON-ARABIAN HORSE BREEDS

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ABSTRACT

Arabian horse breeds are famous for their strength, disease resistance and endurance. Originating from Arabian Peninsula, their progeny is considered among the best horse breeds of the world. Maternally inherited mitochondrial genome represents high genetic diversity in modern horse population. ATP6 gene of mt DNA from 46 horse samples of Middle Eastern Arabian, Western Arabian, mixed (hybrid) Arabians and non-Arabians were sequenced and analyzed to assess the genetic diversity and phylogenetic relationships among them. We have found nine haplotypes in our study. Totally, 99-nucleotide base substitutions were observed with seven variables, which accounted for six transitions and one transversion. Four single nucleotide polymorphismswereobserved in our study. Arabian horse breeds showed high diversity and shared many haplotypes among the population.The observed haplotype diversity and the average evolutionary divergence over all the sequence pairs were 0.8141 and 0.007 respectively.In addition, these datasets may also be useful for strain genotyping, data conservation, effective breeding and individual breed selection for desirable traits.

Key words: Genetic Diversity, Mitochondrial DNA, ATP6 gene, Arabian horse.

INTRODUCTION

There are more than 300 horse breeds and they are employed in varied activities (Ling *et al.,* 2011).The Arabian horse (*Equuscaballus*) is well known for its history, purity, elegance and endurance. It is the most sought after breed to improve upon genetic traits throughout the world (Glazewska, 2010) and has contributed immensely to improve manydesired characteristics among the Thoroughbreds (Bowling and Ruvinsky2000)and the Lipizzan (Zechner *et al.,*2002). It was observed long time ago that different Arabian horse populations or strains bred by the Bedouins are Kehilan (Koheilan, Kuhailan), Seglawi (Saklawi), Abeyan (Obajan), Hamdani and Hadban were of heterogeneous origin (Głażewska, 2010). These breeds are found in Saudi Arabian private farms, few were further hybridized with European racing and performance breeds (Ahmed*et al.,*2011). Initially, the horses were bred for endurance, strength and speed in central Eurasia, 5.5-6k years ago, leading to their early domestication (Outram *et al.,* 2009).

The Domestic horse population exhibits significant mitochondrial DNA (mtDNA) diversity inherited maternally with limited geographic spread (Vila 2001; Jansen *et al.,* 2002; Cieslak *et al.,* 2010; Lippold *et al.,* 2011;Achilli *et al.,* 2012). Early breeder realized that maternal inheritance contributed specifically to the gene flow during domestication (Petersen *et al.,* 2013;Wallner *et al.,* 2013). The traditional observations support the hypothesis that the horses with variation in mtDNA genes may influence performance characteristics (Harrison and Turrion-Gomez 2006). Further studies have shown that the role of mitochondrial genes (mt genes) in the depletion of muscle ATP (adenosine triphosphate) content is far greater with long distance runners compared to short distance ones (Harris *et al.,*1987). These mt genes may further influence the potential and stamina of thoroughbreds. Studies focused on few modern horse breeds clearly illustrates that the paternal inheritance with nuclear DNA analysis shows limited diversity while maternal inheritance had highly significant diversity (Lindgren *et al.,*2004; Ling *et al.,* 2010). Horse breed origin and genetic relationships are mainly analyzed by mitochondrial genome (Royo *et al*., 2005; Aberle *et al*., 2007; Glazewska, 2010), useful in evolution of maternal inheritance and to find out the founder mares of some breeds (Bowling *et al*., 2000; Hill *et al*., 2002). Haplotype analysis of mtDNA provides relevant informationabout the history and genetic diversity in autochthonous horse populations (Aberle *et al.,* 2007; Kakoi *et al.,* 2007). The frequent rate of mutations result in increase of haplotype diversity which helpful in evaluation of haplogroup diversity. The

mtDNA shows elevated rate of mutations when compared to the nuclear DNA due to absence of DNA repair and proof reading mechanism that leads to higher nucleotide base changes. The polymorphism rate is greater between individual mtDNA and would be a useful tool for diversity analysis (Brown *et al.,* 1979). In addition, it is widely used for phylogenetic analysis of intra and inter species (Mirol *et al.,* 2002) and to characterize intra breed variation (Glazewska, 2010; Bowling *et al.,* 2000).

The aim of this study was to sequence the partial fragment of mt gene ATP6 from various horse breeds of Saudi Arabiato analyze genetic diversity and to assess phylogenetic relationships among them.

MATERIALS AND METHODS

Sample Collection and Genomic DNA Extraction: All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the King Abdulaziz University (Reference No. 298-14 Animal study,10 November 2014). Blood samples were collected from 46 horses of various breeds from private farms of Jeddah and were grouped under Saudi(14), Arabian(13), English(4), Indian(4) and Hybrid(10) categories. Blood was taken from the jugular vein into labelled heparinized tubes, which were kept on ice until storage in lab at -20°C. DNA was isolated from 0.2ml blood samples by QIAGEN DNA (Cat. No. 51104, Hilden, Germany) extraction kit, according to manufacturer's protocol. DNA was quantifiedon spectrophotometer(JENWAY, Genova Nano, UK)and was used for polymerase chain reaction (PCR).

PCR Amplification and Sequencing: The primers were design based on previously published sequences in the GenBank(NC_001640) (Xiufeng and Árnason 1994). The primersi. eForward 5′- CTATGGGCAGGGACAGTATT -3′ and Reverse 5′- AAAGGCTTACCAGGAGAGTG -3′were used to amplify the fragment between 8285 and 8605. Primers used for gene amplification and sequencing were synthesized at Macrogen Inc, Korea. PCR was carried out in a 25µL reaction mixture containing,5 µL Jena Bioscience *Taq* PCR Master Mix (*Taq* DNA Polymerase, PCR Buffer, $MgCl_2$, and dNTPs), 2 µL DNA (100ng) template, 10 picomoles as a final concentration of each primer and distilled water to final volume of 25µL. The PCR program for ATP6 gene was set for 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min and extension at 72°C for 1 min. This amplification program was run in a Thermal cycler (MULTIGENE, Labnet International Inc., NJ, US). PCR products were resolved on 1% agarose gel electrophoresis, and visualizedwith ethidium bromide staining, and confirmed the amplicons of 320 bp size with 100 bp DNA ladder (Fig. 1) under UV illuminator. The PCR products were sent to MacrogenInc., Korea for sequencing.

Sequence Data Analyses: The sequence data edited and aligned in codon code aligner V. 5.0.1(Codoncode.com) software to find out the variable sites. The forward and reverse primer sequencing results were evaluated with the reference sequence to eliminate the gaps and missing data. This provided us with the accurate 320bp nucleotide sequence from each sample. All the 46 samples were analyzed and compared with the reference sequence (NC_001640). The sequences were aligned with ClustalW multiple alignment tool (Larkin *et al.,* 2007). Number of Haplotypes and Haplotype diversity were obtained with DNAsp5 software (Librado and Rozas, 2009).

We reconstructed the Neighbor-Joining Phylogenetic tree based on the Tamura Nei model with Geneious® 8.0.5 software (Kearse *et al.,* 2012). Neighbor-Joining consensus tree constructed with 1000 bootstrap replicate values. The following mtDNA sequences of *Equusprzewalskii* (NC_024030.1) *Equusprzewalskii* (NW_007678569.1) *Equusburchellii* (JX312729.1), *Equuscaballus* (KF038160.1), *Equuscaballus* (AP013078.1) were used in phylogenetic tree to elucidate the relationship with present study population and the *Equusasinus*(X97337.1) was used as an out-group (Xu Janke and Arnason 1996).

RESULTS AND DISCUSSION

Mitochondrial ATP6 gene partial fragment of 320bp from 46 horse samples of Arabian, non-Arabian and hybrid breeds of *E. caballus* was amplified (Fig. 1) and sequenced. ATP6 gene has been shown to be a reliable marker for evaluating polymorphism in Arabian *E. caballus* breeds (Ahmed *et al.,* 2011).

In Fig. 2, some of the aligned sequences of the gene represented at bottom with asterisk sign (*) are identical and those with no sign are polymorphic ones. The transitional SNP's are highlighted with bright green color and the transversional SNP (A→T) at 273rd position highlighted with yellow in color.

Selected horse sequences used for alignment and the sequences with similar nucleotides resemble with asterisk symbol (*) and those which are polymorphic does not represent with asterisk symbol. The transitional SNP's are highlighted with bright green color and the transversional SNP (A→T) at 273rd position highlighted with yellow in color.

The data clearly showed base frequencies of A= 27.7%, C = 31.7%, G = 14.9%, T= 25.6%. Of these nucleotides, 313 were identical and seven were variables. Seven polymorphic sites were observed including six transitions and one transversion. The samples were 13 Arabian, 14 Saudi, 4 English, 1 German, 4 Indian and 10

Hybrids. Of which 5 variables were observed in Arabian, 5 in Saudi, 3 in English, 4 in German, 6 in Indian, 7 in Hybrid ones. Remarkably all variables existed at least twice and both forward and reverse primer sequencing confirmed the data authenticity.

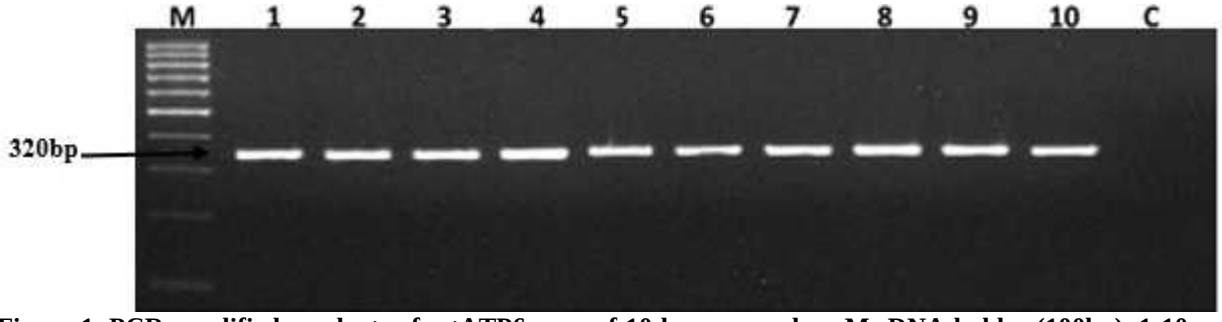

Figure 1. PCR amplified products of mtATP6 gene of 10 horse samples. M- DNA ladder (100bp), 1-10 samples, C- control

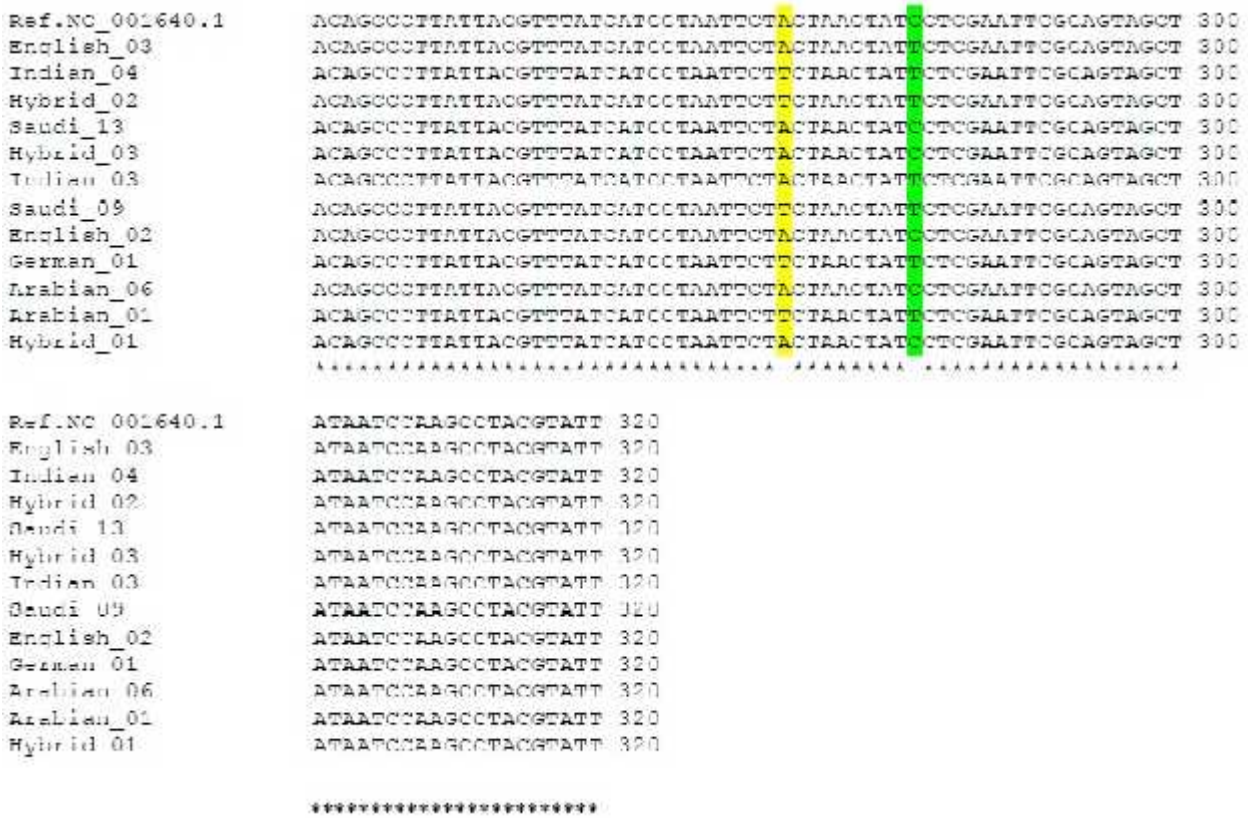

Figure 2. CLUSTAL 2.1 multiple sequence alignment representing the nucleotide changes inATP6 gene of different horse breeds.

Nucleotide sequencing of the mtATP6 gene based on genotypic data submitted to National Center for Biotechnology Information (NCBI) of GenBank database with the accession numbers (KP318510-KP318518). Nine haplotypes were found in tested population with Haplotype diversity 0.8141. In Przewalski's horses when estimated from whole mtDNA resulted 0.54% diversity (Goto *et al.*, 2011), where in Tibetian horse breeds it was 0.66 (Lindgren *et al.*, 2004) and it was 1.6% in mtDNA control region of Przewalski's haplotypes (Goto *et al.*, 2011). Maximum population observed in haplotype 5 followed by 2, 1, 3, 6 respectively and remaining haplotypes end up with sample size one as shown in Table 1. Haplotype 2 represented the most number of variable breeds as five and it denotes the mixed population of all breeds. This group has the four polymorphic sites of same origin. Haplotype 5 shown up with three breeds, of which Saudi (7), Arabian (4) and

English (2) grouped along with reference sequence (NC_001640). These populations do not exhibit any of the polymorphism. Haplotype 1 shows four breeds of Arabian (2), Saudi (2), English (1) and Hybrid (4). This group has two polymorphic sites of same origin. Haplotype 3 shows four breeds as well and each breed of Arabian, Hybrid, Indian and Saudi consist of two individuals. This cluster has three polymorphic sites of same origin. Haplotypes 4, 7, 8 and 9 forms dispersed clusters due to their infrequent variables and Haplotype 4 observed with five polymorphic sites (Fig. 3) and recorded as highest one. The large number of haplogroups are based on recurrent mutations, both in coding and non-coding region of the genome, which are useful in the evaluation of modern horse haplogroup diversity and characterize the influence of mt genes on the performance and endurance of horse breeds (Bower *et al.,* 2010;Carelli *et al.,* 2006).

Table 1. Haplotypes found in tested samples with their Gene bank accession numbers

Haplo type	Names with Id number	Gene Bank Accessions	Total
1	Arabian_06 Arabian_08 English_02 Hybrid_03 Hybrid_06 Hybrid_07 Hybrid_09 Saudi_06 Saudi_13	KP318510	9
2	Arabian_01 Arabian_04 Arabian_13 German_01 Indian_04 Hybrid_02 Hybrid_08 Saudi_09 Saudi_10 Saudi_14	KP318511	10
3	Arabian_10 Arabian_11 Hybrid_04 Hybrid_05 Indian_01 Indian_02 Saudi_01 Saudi_03	KP318512	8
4	Indian_03	KP318513	1
5	Arabian_02 Arabian_03 Arabian_05 Arabian_07 English_01 English_04 Saudi_02 Saudi_04 Saudi_05 Saudi_07 Saudi_08 Saudi_11 Saudi_12	KP318514	13
6	Arabian_12 English_03	KP318515	2
7	Arabian_09	KP318516	1
8	Hybrid_10	KP318517	1
9	Hybrid_01	KP318518	1
			46

Haplotype 5 (KP318514) accounts for large sample size and reflect to the reference sequence (NC_001640).

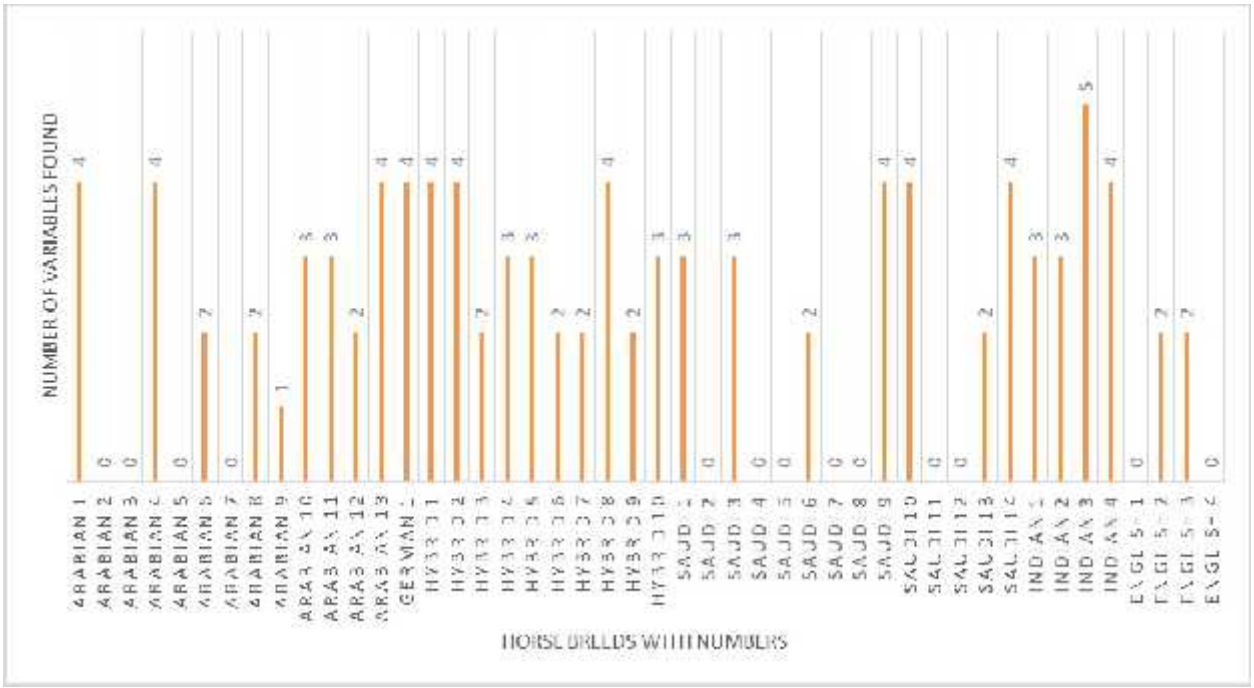

Figure 3. The chart showing 99 nucleotide base substitutions and the number of polymorphic sites among different horse breeds

Seven different polymorphic sites observed in this study and interestingly they have existed at least twice, confirming the sequencing accuracy. The polymorphism of the 320bp representing the partial sequence of the ATP6 gene in 46 individual horses of Arabian, non-Arabian and hybrid breeds, as illustrated with variable number (Fig. 3).

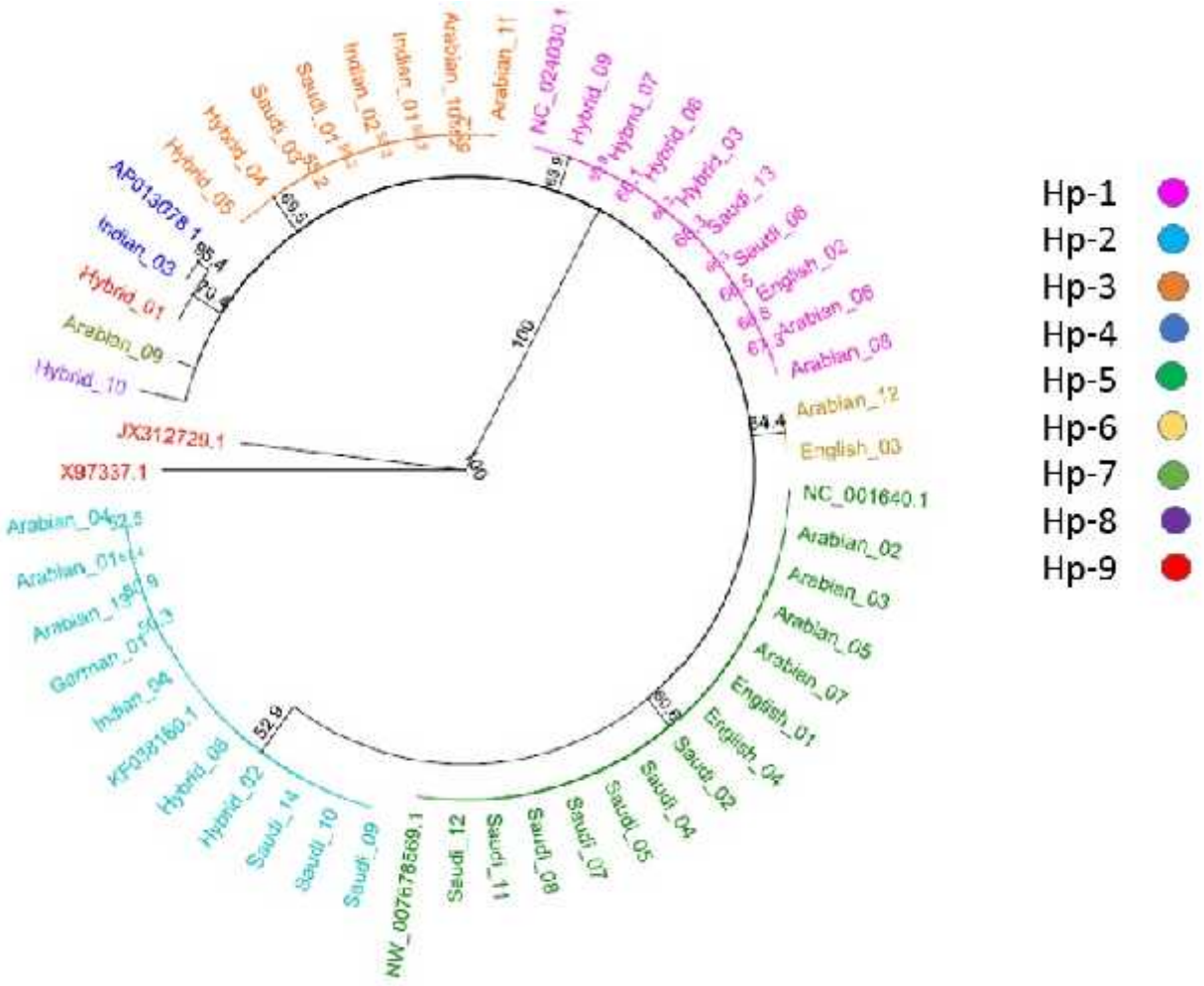

Figure 4. The phylogenetic tree constructed by using Neighbour-Joining method and rooted with the reference sequence *Equusasinus* (X97337.1). Each haplotype represented with different color and bootstrap value. The accession numbers for displayed haplotypes are (KP318510-KP318518)

Fig. 4displays the consensus Neighbor-Joining tree with nine haplotypes (Kearse *et al.,* 2012). The tree has seven clades and an out group *Equusasinus* (X97337.1) along with sub out group *Equusburchellii* (JX312729.1). The thoroughbreds mtDNA sequences of *Equusprzewalskii* (NC_024030.1) found with Haplotype 1 and *Equusprzewalskii* (NW_007678569.1) found with Haplotype 5 used in tree construction as a reference data. Some other reference sequences from NCBI also used which can be found in other Haplotypes. Each haplotype represented with a different color and with reasonable bootstrap value.

The Arabian individuals found in Haplotype 1, 2, 3, 5, 6 and 7 showing maximum diversity were further stratified. This population was the most variable due to its individuals spread among many other clades. The Saudi individuals were found in Haplotype 1, 2, 3 and 5. English population showed close relationship to Arabian and Saudi Haplotypes (1, 5 and 6). The non-Arabian population canbe found along with Arabian groups but detached also; for example Haplotype 4. Horses exhibit long-range haplotype sharing among them (Wade *et al.,* 2009). Horse mitochondrial genome shows greater haplogroups diversity within the domestic animals, as previously reported for taurine cattle (Groeneveld *et al.,* 2010;Zeder 2008). Our study and previous reports have clearly established that the haplotypes of domestic horses are much more widespread (Achilli *et al.,* 2012).

Table 2. Various SNP's found in studied horse breeds and their frequencies

SNP	Change	Type	Position	Variant Frequency
A	G→A	Transition	75	70.20%
A	G→A	Transition	78	42.60%
T	A→T	Transversion	273	40.40%
T	C→T	Transition	282	29.80%

The transversional SNP (A→T) at 273rd position with variant frequency 40.40% is the significant one.

The SNP data was generated by using Geneious® 8.0.5 (Kearse *et al.,* 2012)software as shown in the Table 2. The SNP A at 75th position shows highest transitional variant frequency of 70.20% followed by SNP A 42.60% and SNP T 29.80% at 78 and 282 positions respectively. The transversional SNP T shows 40.40% of variant frequency. Transversional SNP change (A→T) found at 273rd position of the gene and remaining were all transitional changes. This has been reported previously, but all other were different (Ahmed *et al.,* 2011). This one can be foundin Haplotypes 2, 3, 6 and 8 (KP318511, KP318512, KP318515 and KP318517). The other variables found at 96, 207, 219 positions has not been considered as the valid SNP's due to their low frequencies among the tested samples.

The average evolutionary divergence over all the sequence pairs was found to be 0.007. The number of base substitutions per site from averaging over all the sequence pairs. Estimates of average evolutionary divergence over sequence pairs within groups were calculated and the number of base substitutions per site from averaging over all sequence pairs within each group are shown in Table 3.

Table 3. The average evolutionary divergence over sequence pairs within different horse groups.

Breed Name	Diversity
Saudi	0.007
Arabian	0.007
English	0.005
Indian	0.007
Hybrid	0.008
German	n/c

As anticipated hybrid breeds were shown to be more diverse due to cross breeding, non-Arabian showed less diversity and Arabian showed intermediate diversity with respect to the studied population. Analyses were conducted using the Maximum Composite likelihood model (Tamura *et al.,* 2004). The presence of n/c in the results denotes case in which it was not possible to estimate evolutionary distances due to low number of samples.

Conclusion: The present study was carried out to evaluate the genetic diversity amongst different Arabian and non-Arabian horsebreeds. Most of the Arabian and Saudi horse breeds revealed close genetic relationship. Arabian horse population represented significantly greater genetic diversity as they have had shared many haplotypes. Nine haplotypes and four SNP'swere found in our study.These comparative analyses of ATP6 gene among studied breeds mayfurther aid in detecting breeds for performance and endurance. Future studies on a number of breeds with large sample size will help in creating valuable genetic database for future breeding strategies and maintenance of diversity.

Acknowledgments: This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, under the grant no. 9-130-35-GR. The authors therefore, acknowledge with thanks DSR for technical and financial support.

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