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SNP mapping and phylogenetic analysis of Saudi Arabian horse breeds based on mitochondrial genome sequencing

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Arabian horse breeds (*Equus caballus* L.) are renowned for elegance, endurance and their contribution to Thoroughbreds. In heredity, the mitochondrial (mt) genome is maternally inherited and represents extensive genetic diversity. The mt genomes of Seglawi and Hadban horse breeds of Saudi Arabia were sequenced to decipher the genetic variations in the coding and non-coding regions. We observed that the coding region of mt genome has 11 and 34 amino acid variations in Seglawi and Hadban breeds, respectively. Sequencing analyses of *COX1* gene indicated highest variations of which, 5 in Seglawi and 8 in Hadban followed by the *NADH5* gene. The mitochondrial genes of respiratory chain showed positive selections with respect to different environments. Our data also highlighted that the Hadban breed had much higher nucleotide changes as compared to Seglawi and together they formed individual branches in phylogenetic tree. However, the tree shows that they were relatively branched to Arabian horse breeds. This study on two Arabian horses shed light on variations among mt genes and their phylogenetic relationship with other horse breeds.

Keywords: *Equus caballus*, Genetic variation, Hadban, Positive selections, Seglawi, Stallions

Arabian horses have contributed immensely towards the improvement of Spanish and Lipizzan horse breeds[1,2]. In the 18th century, the Lipizzan breed was largely bred by Arabian horses[2]. The Seglawi one of the Arabian breeds formed a separate line out of seven Arabian stallions in Lipizzan breed and contributed 24% of their populations during the period 1810-1920. There were 45 original Arabian and 24 Shagya Arabian horse breeds contributing towards the Lipizzan founder population[2]. Seven classical Arabian origin mares were established (Rozca, Khelil Missaid, Mersucha, Gidrane, djebrin, Mercurio and Theodorosta) and four of them still exist in Lipica stud[3].

The mtDNA plays important role in cellular energy metabolism and maternal inheritance. Its significance in evolutionary analysis, genetics and environmental effects prediction and deducing maps of phylogenetics are extensively documented[4-9], and it serves as an essential genetic tool for addressing domestication process due to its maternal inheritance, lack of recombination and genetic diversity[10-13]. The control region of mtDNA has been extensively studied for its maternal inheritance, haplotype diversity and elevated mutation frequency data[14-16]. The control region is hypervariable and is of small size to explore the variations among populations and phylogenetic analysis of large samples[17-20]. However, the whole mtDNA genomes represented with greater diversity and their mito-chondrial haplogroups are distributed worlwide[10,21-23]. Earlier studies on the mitochondrial genes of respiratory chain have shown positive selections with respect to different environmental conditions in primates[24-26]. The genes with adaptive environment have higher mutations rate and hence the positive selections occur frequently[27]. It may be due to migration of these animals to extreme climatic conditions and other selection pressures. One of studies has reported that the mt genes *ATP6*, *COX3*, *NADH6*, *cytb*, *COX1* and *NADH5* changes were observed with respect to diverse environments[8]. Similarly, another study reported that the *COX1* gene showed positive selection to the Tibetan environments[28].

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In our present study, we attempted to sequence the whole mt genome and compile datasets of genetic variations both in coding and non-coding regions of two horse breeds Seglawi and Hadban. In addition, we performed phylogenetic and Single Nucleotide Polymorphism (SNP) analyses to explore the similarities between these two breeds and genetic relationship with other horse breeds.

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). Two Blood samples were collected from each of two horse breeds including Seglawi and Hadban from private farms of Jeddah. 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 0.2 mL blood samples by QIAGEN DNA (Cat. No. 51104; Hilden, Germany) extraction kit, according to manufacturer's protocol. DNA was quantified spectrophotometrically and was used for polymerase chain reaction (PCR).

PCR amplification and sequencing

The primers were deduced from the horse mtDNA sequence [X79547][29] (Table 1). PCR was carried out in a 25 µL reaction mixture containing; 5 µL Jena Bioscience *Taq* PCR Master Mix (*Taq* DNA polymerase, PCR buffer, $MgCl_2$, and dNTPs), 2 µL DNA template of 100 ng, 10 picomoles as a final concentration of each primer and distilled water up to the volume of 25 µL. The PCR program set for 34 cycles of denaturation at 95°C for 30 s, annealing at 56-58°C for 30 s, initial extension at 72°C for 90 s (primers 9 and 10 for 150 s) and final extension at 72°C for 10 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 visualized with ethidium bromide staining and the size of the amplicons were confirmed using 1kb DNA ladder, under a UV illuminator. The PCR products were sent to Macrogen Inc., Korea for sequencing.

Table 1—The PCR primers to amplify mitochondrial genome

Primer sequences	Position on mitochondrial genome
F: TAACATGAATCGGCGGACA	15,191
R: TTGCTGAAGATGGCGGTAT	706
F: ATTTCCATAGACAGGCATCC	16,599
R: TCACCTCTACCTACGAATCTTCT	1,439
F: CGTAAGGGAACGATGAAAGAT	1,232
R: AATAGATAGAAACCGACCTGGAT	2,565
F: ACGAGAAGACCCTATGGAGC	2,169
R: GTGGAATAGGTTAGTCGTATGTAG	4,835
F: TACCTCTAACCACTACACTAATC	4,646
R: CATAATGGAAATGTGCTACTA	6,499
F: CGAGCATACTTCACATCAGC	6,265
R: TAGCGAAAGAGGCGAATAGA	7,995
F: GACTTTACTACGGTCAATGCTC	7,616
R: TTTCCTTCTATTAGGCTATGGT	9,108
F: CCACCCACAGGTATCCAC	8,993
R: GATGAGGACGGCTACGAT	11,113
F: GAGGCTACGGAATACTACGAA	10,992
R: TAAAGGATTGCTTGAAGGG	12,303
F: GCTAACAACCTTTTCCAACTG	12,182
R: GACCAGGGTAATGTGCGATA	15,359

[F: forward primer; R: reverse primer]

Sequence data analyses

The sequence data edited and aligned in codon code aligner version 5.0.1[30] software to find out the variable sites. For the base calling, quality value considered not less than 20. The forward and reverse primer sequencing results were evaluated with the reference sequence to eliminate the sequencing errors and missing data. This provided us with the precise nucleotide sequence from each sample. A Neighbor joining phylogenetic tree was built using Tamura-Nei method[31]. The sequences were clustered with 1000 bootstrap replicates and the tree was constructed with evolutionary distances based on number of base substitutions per site by using Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6 program)[32,33].

Results

The mitochondrial genomes of the two Saudi horse breeds Hadban and Seglawi were amplified in various lengths of overlapping fragments (Fig. 1) and sequenced data submitted to National Center for Biotechnology Information (NCBI) for which obtained the Genbank accession numbers MK100122 and MK100123, respectively. We observed 60 and 89 nucleotide substitutions in the coding and non-coding region (transitions, transversions, insertions and deletions) in Seglawi and Hadban breeds, respectively. Table 2 shows nucleotide variations in coding genes of mtDNA. The HRS (Horse Reference Sequence, JN398377) proposed by Achilli *et al*., (2012)[34] and X79547 were used as reference sequences. The HRS (JN398377) differs with X79547 at 357 and 358 nucleotide positions with C nucleotide. The nucleotide positions 2227, 5240, 5279 and 15388

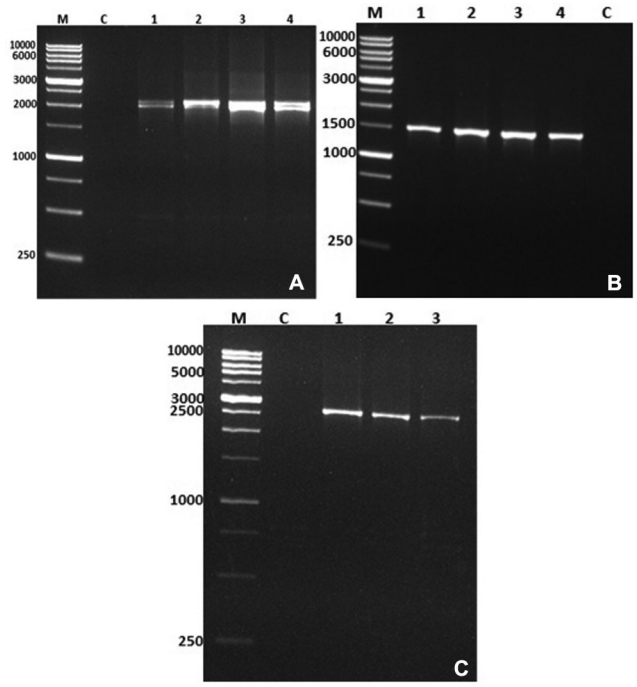

Fig. 1—Horse mitochondrial PCR amplicons of various overlapping spans. (A) Amplicons of 2500 bp with primer set 1; (B) Amplicons of 1500 bp with primer set 2; and (C) Amplicons of 2000 bp with primer set 4. [M, 1 kb DNA ladder; C, Negative control; and 1-4 are samples]

Table 2—Nucleotide variations between Hadban and Seglawi breeds

Variations	Hadban	Seglawi
Transitions	71	44
Transversions	16	9
Deletions	2	2
Insertions	0	5
Total	89	60

were excluded from the assessment due to non-coding region however, represented when compared with X79547 in bold letters in Tables 3 and 4.

Protein coding region

The protein coding genes displayed 55 amino acid modifications in Seglawi (11) and Hadban (34) when compared to JN398377[34] as shown in Table 3. Hadban showed high variations than Seglawi. There were 33 variations in non-coding region (rRNA, tRNA and Misc feature) also comprising of 17 in Seglawi, and 18 in Hadban (Table 4). There were some amino acid changes showed in bold letters, which were according to the reference X79547 in Tables 3 and 4.

Phylogenetic analysis

A Neighbor-joining phylogenetic tree was constructed with Seglawi and Hadban breeds along with some mt genomes of horses from GenBank with MEGA6[32] as shown in Fig. 2. In total, the tree was constructed with 24 horse mitochondrial sequences obtained from GenBank along with *Equus asinus* (X97337.1) as outgroup.

Table 3—Amino acid changes in Seglawi and Hadban horse breeds with respect to reference JN398377 and X79547

Gene	Position Seglawi	JN398377		Position Hadban	JN398377	
NADH1	3307	Pro	His	3374	Lys	Glu
				3380	Lys	Glu
				3386	Lys	Glu
				3507	Leu	His
NADH2		NC			NC	
COX1	5529	Leu	Ser	5529	Leu	Ser
	5829	Leu	Pro	5829	Leu	Pro
	5886	Leu	Pro	5886	Leu	Pro
	6309	Pro	Leu	6309	Pro	Leu
	6532	Ile	Met	6507	Pro	Leu
				6714	Thr	Ile
				6777	Ser	Phe
				6786	His	Arg
COX2		NC		7245	Gln	Arg
				7614	Leu	Gln
				7668	Cys	Tyr
ATP8		NC		7902	Thr	Ile
ATP6		NC		8007	Asn	Ser
				8240	His	Tyr
				8360	Asn	Asp
				8363	Thr	Ala
				8558	Ser	Thr
				8567	Ser	Pro
COX3		NC			NC	
NADH3		NC		9777	Leu	Pro
NADH4L		NC		10112	Arg	Cys
NADH4	11417	Ser	Pro	**10829**	**Leu**	**Val**
	11432	Tyr	His			
	11546	Val	Ile			
NADH5	11882	Thr	Ala	11791	Ile	Met
	11969	His	Tyr	11844	Ser	Leu
				11861	His	Tyr
				11877	Tyr	Phe
				11898	Ile	Thr
				11987	Val	Ile
				12136	Leu	Phe
				12335	Ser	Gly
				12407	His	Tyr
NADH6		NC			NC	
cytb		NC			NC	

[Nucleotide changes in bold are according to X79547. NC, No change]

Discussion

Extensive variations observed in the genomes of mammalian mitochondria within and across the species due to oxidative conditions and susceptible repairing mechanism[35]. In the gene *NADH1*, there is one amino acid change from Pro-His in Seglawi whereas in Hadban there were four amino acid

Table 4—Variations in non-coding region of Seglawi and Hadban breeds

Nucleotide Position	Product	Seglawi	JN398377	Nucleotide Position	Product	Hadban	JN398377
158	rRNA	G	A	19	tRNA	A	T
356	rRNA	C	T	112	rRNA	A	T
358	**rRNA**	**C**	**-**	156	rRNA	A	T
597	rRNA	T	G	158	rRNA	G	A
599	rRNA	A	-	235	rRNA	C	A
601	rRNA	A	T	339	rRNA	C	T
739	rRNA	C	T	356	rRNA	C	T
860	rRNA	G	A	**358**	**rRNA**	**C**	**-**
961	rRNA	C	T	513	rRNA	C	A
1386	rRNA	-	T	739	rRNA	C	T
1683	rRNA	C	A	860	rRNA	G	A
2227	**rRNA**	**-**	**T**	961	rRNA	C	T
5098	**rRNA**	**A**	**T**	1382	rRNA	T	C
5240	**tRNA**	**-**	**A**	1386	rRNA	-	T
5279	**tRNA**	**-**	**A**	1685	rRNA	C	A
7004	tRNA	A	G	**2229**	**rRNA**	**-**	**T**
11693	tRNA	T	C	2510	rRNA	-	T
15390	tRNA	-	T	**5098**	**rRNA**	**A**	**T**
15497	MF	C	T	**5240**	**tRNA**	**-**	**A**
15722	MF	A	G	**5279**	**tRNA**	**-**	**A**
15772	MF	T	C	7003	tRNA	A	G
15828	MF	G	A	11684	tRNA	T	C
				11726	tRNA	G	A

[Nucleotide changes in bold are according to X79547. -, Deletion; MF, Misc Feature]

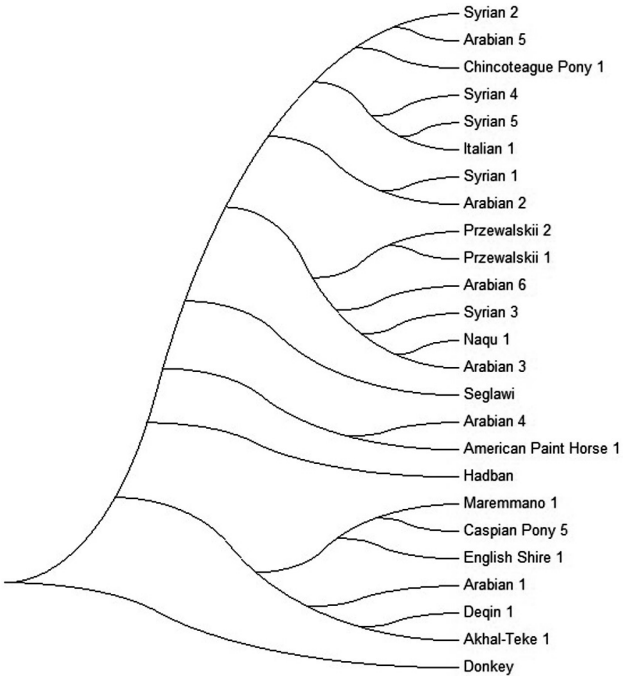

Fig. 2—A Neighbor-joining Phylogenetic tree of Seglawi and Hadban horse mt genomes along with other GenBank sequences and Donkey (*Equus asinus*) (GenBank accession no. X97337.1) as outgroup

modifications when compared to JN398377. There were no mutations in *NADH2* gene in both breeds. In *COX1* gene, there were five amino acid alterations in Seglawi and eight in Hadban. Interestingly, four of them were identical in both breeds i.e., Leu-Ser at 5529, Leu-Pro at 5829, Leu-Pro at 5886, Pro-Leu at 6309 nucleotide positions (Table 3). Our data have shown that there was no SNP in *COX2* of Seglawi however, there were three amino acid changes in Hadban. In case of *ATP8* gene, Hadban breed showed with single amino acid change only. In *ATP6* gene, there were no mutations in Seglawi breed where as in Hadban breed six amino acid changes were observed which includes one of the reported SNP of *ATP6* gene of Arabian horse breeds[36]. In *NADH3* and *NADH4L* genes, only Hadban breed showed Leu–Pro and Arg-Cys amino acids changes at 9777 and 10112 nucleotide positions, respectively. *NADH4* gene had three modifications of amino acids in Seglawi while in Hadban breed, there were no mutations as compared to JN398377, but with reference to X79547 there was a single amino acid change i.e., Leu-Val at 10829 nucleotide position. The gene *NADH5* comprising of two amino acid changes in Seglawi and nine in Hadban. In Seglawi, His-Tyr variation was detected at nucleotide position 11969 whereas in Hadban it was found at 11861 and 12407. The gene *NADH6* did not show any mutations among the studied breeds. Finally, *cytb* gene there were no predictable mutations nor heteroplasmy observed in both breeds[37]. The mitochondrial genes influence the pathway related to energy metabolism and plays

important role in animal adaptation to the particular environmental conditions. Similarly, our data showed changes at nucleotide level between both the breeds in tRNA, rRNA and other related genes. They were according to the reference JN398377 and the changes, which were in bold letters specified according to the reference X79547.

A neighbor-joining phylogenetic tree was constructed with Seglawi and Hadban horses along with some mt genomes of horses from GenBank[31] (Fig. 2). The evolutionary distance obtained was 0.1032 which was computed by using Tamura-Nei method[33]. In total, the tree was built with 24 horse mitochondrial sequences along with *Equus asinus* (GenBank accession no. X97337.1) as outgroup. The phylogenetic analysis was performed by using MEGA6[32]. Hadban and Seglawi horses split into individual branches. Seglawi horse lie in between Arabian and American paint horse branch and the Przewalskii, Arabian, Syrian and Naqu horse branch. The Hadban horse breed relatively branched with the Seglawi and among the largest branch of Maremmano, Caspian pony, English Shire, Arabain, Deqin and Akal-Teke horses at the bottom and Arabian and American paint horse clade on the upper side. The genetic variation observed between the Seglawi and Hadban might be due to the mixed ancestral population. This study on two Arabian horse breeds shed light on the variations of mt genes and their phylogenetic relationship with other horse breeds. Further study with large number of sample data from genetically diverse Arabian breeds and metabolic pathway analysis would provide more information in this regard.

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References

1 Clutton-Brock J, *A Natural History of Domesticated Mammals*. (Cambridge University Press, London), 1999, 65.
2 Dovc P, Kavar T, Solkner H & Achmann R, Development of the Lipizzan Horse Breed. *Reprod Domest Anim*, 41 (2006) 280.
3 Nurenberg H, *Der Lipizzaner: Mit Einem Anhang uber den Kladruber*. (Westarp Wissenschaften, Magdeburg), 1993, 250.
4 Blier P, Natural selection and the evolution of mtDNA-encoded peptides: evidence for intergenomic co-adaptation. *Trends Genet*, 17 (2001) 400.
5 Gray M, Mitochondrial Evolution. *Science*, 283 (1999) 1476.
6 Kivisild T, The Role of Selection in the Evolution of Human Mitochondrial Genomes. *Genetics*, 172 (2005) 373.
7 Raina S, Evolution of base-substitution gradients in primate mitochondrial genomes. *Genome Res*, 15 (2005) 665.
8 Ruiz-Pesini E, Effects of Purifying and Adaptive Selection on Regional Variation in Human mtDNA. *Science*, 303 (2004) 223.
9 Zheng X, Arbogast BS & Kenagy GJ, Historical demography and genetic structure of sister species: deermice (Peromyscus) in the North American temperate rain forest. *Mol Ecol*, 12 (2003) 711.
10 Der Sarkissian C, Ermini L, Schubert M, Yang MA, Librado P, Fumagalli M, Jonsson H, Bar-Gal GK, Albrechtsen A, Vieira FG & Petersen B, Evolutionary genomics and conservation of the endangered Przewalski's horse. *Curr Biol*, 25 (2015) 2577.
11 Yoon SH, Lee W, Ahn H, Caetano-Anolles K, Park KD & Kim H, Origin and spread of Thoroughbred racehorses inferred from complete mitochondrial genome sequences: Phylogenomic and Bayesian coalescent perspectives. *PloS ONE*, 13 (2018) e0203917.
12 Cardinali I, Lancioni H, Giontella A, Capodiferro MR, Capomaccio S, Buttazzoni L, Biggio GP, Cherchi R, Albertini E, Olivieri A & Cappelli K, An overview of ten Italian horse breeds through mitochondrial DNA. *PloS ONE*, 11 (2016) e0153004.
13 Librado P, Gamba C, Gaunitz C, Der Sarkissian C, Pruvost M, Albrechtsen A, Fages A, Khan N, Schubert M, Jagannathan V & Serres-Armero A, Ancient genomic changes associated with domestication of the horse. *Science*, 356 (2017) 442.
14 Hutchison C, Newbold J, Potter S & Edgell M, Maternal inheritance of mammalian mitochondrial DNA. *Nature*, 251 (1974) 536.
15 Ishida N, Hasegawa T, Takeda K, Sakagami M, Onishi A, Inumaru S, Komatsu M & Mukoyama H, Polymorphic sequence in the D-loop region of equine mitochondrial DNA. *Anim Genet*, 25 (1994) 215.
16 Ishida N, Oyunsuren T, Mashima S, Mukoyama H & Saitou N, Mitochondrial DNA sequences of various species of the genus Equus with special reference to the phylogenetic relationship between Przewalskii's wild horse and domestic horse. *J Mol Evol*, 41 (1995).
17 Lister A, Kadwell M, Kaagen LM, Richards MB & Stanley HF, Ancient and modern DNA in a study of horse domestication. *Ancient Biomol*, 2 (1998) 267.
18 Vila C, Widespread Origins of Domestic Horse Lineages. *Science*, 291 (2001) 474.
19 Othman OE, Mahrous KF & Shafey HI, Mitochondrial DNA genetic variations among four horse populations in Egypt. *J Genet Eng Biotechnol*, 15 (2017) 469.
20 Nezhad SRK, Modheji E & Zolgharnein H, Polymorphism analysis of mitochondrial DNA control region of hawksbill turtles (Eretmochelys Imbricata) in the Persian Gulf. *Indian J Geo Mar Sci*, 42 (2013) 300.
21 Librado P, Fages A, Gaunitz C, Leonardi M, Wagner S, Khan N, Hanghøj K, Alquraishi SA, Alfarhan AH, Al-Rasheid KA & Der Sarkissian C, The evolutionary origin and genetic makeup of domestic horses. *Genetics*, 204 (2016) 423.
22 Schubert M, Jónsson H, Chang D, Der Sarkissian C, Ermini L, Ginolhac A, Albrechtsen A, Dupanloup I, Foucal A, Petersen B

& Fumagalli M, Prehistoric genomes reveal the genetic foundation and cost of horse domestication. *Proc Nat Acad Sci USA*, 111 (2014) E5661.

23 Librado P, Der Sarkissian C, Ermini L, Schubert M, Jónsson H, Albrechtsen A, Fumagalli M, Yang MA, Gamba C, Seguin-Orlando A & Mortensen CD, Tracking the origins of Yakutian horses and the genetic basis for their fast adaptation to subarctic environments. *Proc Nat Acad Sci USA*, 112 (2015) E6889.

24 Schmidt T, Goodman M & Grossman L, Molecular evolution of the COX7A gene family in primates. *Mol Biol Evol*, 16 (1999) 619.

25 Wu W, Schmidt T, Goodman M & Grossman L, Molecular Evolution of Cytochrome c Oxidase Subunit I in Primates: Is There Coevolution between Mitochondrial and Nuclear Genomes? *Mol Phylogenet Evol*, 17 (2000) 294.

26 Wu W, Goodman M, Lomax M & Grossman L, Molecular Evolution of Cytochrome c Oxidase Subunit IV: Evidence for Positive Selection in Simian Primates. *J Mol Evol*, 44 (1997) 477.

27 Xu S, Luosang J, Hua S, He J, Ciren A, Wang W, Tong X, Liang Y, Wang J & Zheng X, High altitude adaptation and phylogenetic analysis of Tibetan horse based on the mitochondrial genome. *J Genet Genomics*, 34 (2007) 720.

28 Xu SQ, Yang YZ, Zhou J, Jing GE, Chen YT, Wang J, Yang HM, Yu J, Zheng XG & Ge RL, A mitochondrial genome sequence of the Tibetan antelope (*Pantholops hodgsonii*). *Genomics Proteomics Bioinformatics*, 3 (2005) 5.

29 Xiufeng X & Arnason U, The complete mitochondrial DNA sequence of the horse, *Equus caballus*: extensive heteroplasmy of the control region. *Gene*, 148 (1994) 357.

30 Sequence Assembly and Alignment with CodonCode Aligner version 5.0.1. Available at: http://www.codoncode.com/aligner/. (2014) Accessed on February 12, 2016.

31 Saitou N & Nei M, The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, 4 (1987) 406.

32 Tamura K, Stecher G, Peterson D, Filipski A & Kumar S, MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol*, 30 (2013) 2725.

33 Tamura K & Nei M, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, 10 (1993) 512.

34 Achilli A, Olivieri A, Soares P, Lancioni H, Kashani BH, Perego UA, Nergadze SG, Carossa V, Santagostino M, Capomaccio S, Felicetti M, Al-Achkar W, Penedo MCT, Verini-Supplizi A, Houshmand M, Woodward SR, Semino O, Silvestrelli M, Giulotto E, Pereira L, Bandelt HJ & Torroni A, Mitochondrial genomes from modern horses reveal the major haplogroups that underwent domestication. *Proc Nat Acad Sci USA*, 109 (2012) 2449.

35 Gibson A, Gowri-Shankar V, Higgs PG & Rattray M, A comprehensive analysis of mammalian mitochondrial genome base composition and improved phylogenetic methods. *Mol Biol Evol*, 22 (2005) 251.

36 Ahmed MM, Sheikh A, Mutwakil MH, Saini KS, Alsulaimany FA, Hanafy AA & Sabir JS, Comparative analysis of ATP6 mitochondrial gene diversity in Arabian and non-Arabian horse breeds. *J Anim Plant Sci*, 26 (2016) 437.

37 Zhao Q, Kang Y, Pu Y, Niu L, Guan W, He X, Zhang H, Lim H, Ma Y & Zhong T, Primer effect in the detection of mtDNA heteroplasmy: insights from horse *Cytochrome b* gene. *Mitochondrial DNA*, 26 (2015) 178.