

Genetic characterization of Kushum horses in Kazakhstan based on haplotypes of mtDNA and Y chromosome, and genes associated with important traits of the horses

Trung B. NGUYEN^{1,2}, Ripon C. PAUL^{1,3}, Yu OKUDA^{1,4}, Thu N. A. LE^{1,5}, Phuong T. K. PHAM², Kushaliye J. KAISSAR⁶, Akhmedenov KAZHMURAT⁷, Sarsenova BIBIGUL⁷, Meirat BAKHTIN⁸, Polat KAZYMBET⁸, Suleimenov Zh MARATBEK⁹, Alikhan MELDEBEKOV⁹, Masahide NISHIBORI¹⁰, Takayuki IBI¹, Takehito TSUJI¹ and Tetsuo KUNIEDA^{1,11*}

¹Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan

²An Giang University, Vietnam National University, An Giang, Vietnam

³Patuakhali Science and Technology University, Barishal, Bangladesh

⁴Okayama University of Science, Okayama 700-0005, Japan

⁵University of Agriculture & Forestry, Hue University, Hue, Vietnam

⁶Zhangir Khan West Kazakhstan Agrarian-Technical University, Uralsk, Kazakhstan

⁷M. Utemisov West Kazakhstan State University, Uralsk, Kazakhstan

⁸Radiobiological Research Institute, JSC Astana Medical University, Astana, Kazakhstan

⁹Institute of Zoology, Almaty, Kazakhstan

¹⁰Graduate School of Integrated Sciences for Life, Hiroshima University, Higashihiroshima 739-8528, Japan

¹¹Faculty of Veterinary Medicine, Okayama University of Science, Imabari 794-8555, Japan

The Kushum is a relatively new breed of horses in Kazakhstan that was established in the middle of the 20th century through a cross between mares of Kazakhstan local horses and stallions of Thoroughbred, Trotter, and Russian Don breeds to supply military horses. To reveal the genetic characteristics of this breed, we investigated haplotypes of mitochondrial DNA (mtDNA) and single-nucleotide polymorphisms of the Y chromosome, as well as genotypes of five functional genes associated with coat color, body composition, and locomotion traits. We detected 10 mtDNA haplotypes that fell into 8 of the 17 major haplogroups of horse mtDNA, indicating a unique haplotype composition with high genetic diversity. We also found two Y-chromosomal haplotypes in Kushum horses, which likely originated from Trotter and/or Don breeds. The findings regarding the mtDNA and Y-chromosomal haplotypes are concordant with the documented maternal and paternal origins of the Kushum horses. The allele frequencies of ASIP, MC1R, and MATP associated with coat color were consistent with the coat color variations of Kushum horses. The allele frequencies of MSTN associated with endurance performance and those of DMRT3 associated with gait suggested that the observed allele frequencies of these genes were the result of selective breeding for these traits. As a result of this study, we were able to obtain useful information for a better understanding of the origin and breeding history of the Kushum horse breed using molecular markers.

Key words: coat color, Kazakhstan, locomotion traits, mtDNA, Y chromosome

J. Equine Sci.
Vol. 31, No. 3
pp. 35–43, 2020

Received: June 26, 2020

Accepted: July 22, 2020

*Correspondence author. e-mail: t-kunieda@vet.ous.ac.jp

©2020 Japanese Society of Equine Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

Kazakhstan is one of the major countries that produce horses and exports horse meat, and many people in Kazakhstan produce koumiss, a popular fermented liquor made from horse milk. Horses are also commonly used for riding, herding, and racing in this country. Therefore, many original horse breeds have been established and raised in different

regions of Kazakhstan. The Kushum is one of the Kazakhstan original horse breeds established in the Ural region of West Kazakhstan (Fig. 1) between 1931 and 1976 through crosses between mares of local horses and stallions of Thoroughbred, Trotter, and Russian Don breeds [5]. The original goal of the breeding of Kushum horses was to improve the body size, endurance performance, and gait of the local horses to match the military demand for war horses before World War II. Then, the Kushum horses have mainly been used for producing milk and meat, as well as for herding cattle and sheep after World War II [5]. The total number of heads of Kushum horse was 4,829 as of 1980 [5]. The average withers heights of Kushum stallions and mares are 159 and 154 cm, respectively [5], and their coat colors are mainly bay and chestnut and occasionally black. Kushum horses are well adapted to herd maintenance in semi-desert pastures of West Kazakhstan. However, the genetic characteristics of this breed, such as the genetic diversity, phylogeny, and allele frequencies of genes associated with important traits of the horse, are not well understood.

Haplotypes of mitochondrial DNA (mtDNA) and Y-chromosomal markers are commonly used for investigating genetic diversity and phylogenetic relations among breeds or populations of animals in maternal and paternal lineages, respectively. Phylogenetic relationships among horse populations as well as the origin and migration of domestic horses have been extensively investigated using mtDNA haplotypes inherited through maternal lineages [1,

13]. Previous studies have indicated remarkable genetic diversity in mtDNA haplotypes among global horse populations, suggesting multiple origins of maternal lineages during horse domestication [22, 29]. Conversely, during the breeding of domestic animals, a limited number of stallions are used, and genetic introgression between breeds or populations is mainly realized through paternal rather than maternal lineages, since effective genetic introgression can be achieved by introducing few stallions. Recently, Y-chromosomal haplotypes have also been used for phylogenetic analyses [6, 31, 32]. Particularly, Wallner *et al.* [31] identified various single-nucleotide polymorphisms (SNPs) of the horse Y chromosome, and these SNPs have been utilized for phylogenetic analysis of the paternal lineage [6, 9, 15].

In horses, coat color is a crucial phenotype that plays an important role in the characterization of specific horse breeds and correct animal identification. The basic coat colors, namely bay, chestnut, and black, are determined by the melanocortin 1 receptor (*MC1R*) and agouti-signaling protein (*ASIP*) genes. Horses homozygous for *MC1R* and *ASIP* mutations show chestnut and black coat colors, respectively, whereas horses harboring the wildtype alleles of both genes show a bay coat color [21, 25]. In addition to these basic coat colors, cream dilution is caused by a mutation of the solute carrier family 45 member 2 (*MATP*) gene. A missense mutation of *MATP* results in a buckskin or palomino coat color in heterozygous horses and a double-dilute coat color in homozygous horses [20].



Fig. 1. A map of Kazakhstan indicating the sampling locations (Zhanibek and Kaztal) and the region where the Kushum horse was established (Ural).

Since horses are mainly used because of their physical performance, breeds have been intensively selected for physical performance-related traits such as locomotion traits. Recently, the genes associated with physical performance of horses have been identified via genome-wide association studies (GWASs) [2, 7, 10, 26–28]. For example, a genetic variant of the myostatin (*MSTN*) gene is associated with the racing performance of horses [11, 27], and a nonsense mutation of the doublesex and mab-3 related transcription factor 3 (*DMRT3*) gene shows a major effect on locomotion patterns of horses, including ambling [2]. Therefore, selective breeding for these physical performance-related traits might result in changes of the allele frequency of these genes in the breeds.

The purpose of this study was to reveal the genetic characteristics of Kushum horses. For this purpose, we investigated the nucleotide sequences of the hypervariable D-loop region of mtDNA and genotypes of Y-chromosomal SNPs to clarify the phylogenetic relationships and genetic diversity of the maternal and paternal lineages of Kushum horses. We also investigated the genotype distributions and allele frequencies of genes associated with coat color and physical performance to understand the genetic composition of Kushum horses related to these traits.

Materials and Methods

Sampling and DNA extraction

A total of 22 blood samples were collected from 7 male and 15 female Kushum horses in the Zhanibek and Kaztal regions of West Kazakhstan (Fig. 1) as a part of field research on native livestock in Central Asian countries conducted by the Society for Researchers on Native Livestock from 2010 to 2019. These horses were selected from local herds of Kushum horses to avoid the sampling of horses sharing kinship. The blood samples were collected from the jugular vein using vacuum tubes containing EDTA in accordance with the ethics regulations of Zhangir Khan West Kazakhstan Agrarian-Technical University. DNA was extracted from whole blood cells according to the standard phenol–chloroform extraction method.

mtDNA haplotype analysis

To determine mtDNA haplotypes, a DNA fragment containing the D-loop region of mtDNA (15,494 to 15,740, 247 bp) was amplified by PCR using primers [10], and the amplified fragments were directly sequenced using the dideoxy method. PCR was performed in a 10 μ l reaction volume containing 10 ng genomic DNA, 0.2 μ M primers (5'-CTAGCTCCACCATCAACACC-3' and 5'-ATGGCCCTGAAGAAAGAACC-3'), 0.2 μ mol/l dNTPs, 2 μ l 5 \times PCR buffer, and 1 U GoTaq DNA

polymerase (Promega, Madison, WI, U.S.A.) under the following conditions: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 60 sec, and elongation at 72°C for 30 sec; and then final extension at 72°C for 10 min. The parameters of the mtDNA sequences, including haplotype number, haplotype diversity, and nucleotide diversity, were calculated using DnaSP 5.10.1 [19]. The obtained sequence data were aligned using ClustalW in MEGA 7.1 [17], and a dataset of 97 haplotypes of the D-loop region, including 10 Kushum horse haplotypes and 87 haplotypes reported by Cieslak *et al.* [3], was generated. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.1 with 1,000 random bootstrap replicates.

Y-chromosomal haplotype analysis

To analyze Y-chromosomal haplotypes, the genotypes of four SNPs, namely rAX, rA, rW, and rD, in the male-specific region of the Y chromosome were determined via direct sequencing of DNA fragments amplified by PCR [9, 31]. These four SNPs were selected from various Y-chromosomal SNPs reported by Wallner *et al.* [31, 32] and Filkel *et al.* [6] to classify the Y-chromosomal haplotypes into five different groups. PCR was performed in a 10 μ l reaction volume containing 10 ng genomic DNA, 0.3 μ M primers (rAX, 5'-CTCTGGCAAACAGTCTGTGA-3' and 5'-CACCTGGCTCCAAAGCTATC-3'; rA, 5'-GGCCTA-AGTTGTTTCGAGAG-3' and 5'-TGACTGGTGGTGTCCAGTGT-3'; rW, 5'-AAAGTGCATCCCAGAAGTGC-3' and 5'-ACCCATCAACATACATACCACTT-3'; and rD, 5'-GTCTACAGTAGGGAGGGTCC-3' and 5'-AGAAGAG-CAGTGCCCATTTGTG-3'), 1 μ mol/l dNTPs, 2 μ l 5 \times PCR buffer, and 1 U of GoTaq polymerase (Promega, Madison, WI, U.S.A.) under the following conditions: initial denaturation at 95°C at 3 min; 35 cycles of denaturation at 95°C for 40 sec, annealing at 52–63°C for 60 sec, and elongation at 72°C for 30 sec; and then final extension at 72°C for 10 min. The PCR products were directly sequenced using the dideoxy method.

Genotyping of genes associated with coat color and locomotion traits

For genotyping of functional genes associated with coat color, body composition, and locomotion traits, genotypes of *ASIP* and *DMRT3* were determined via direct sequencing of DNA fragments amplified by PCR using primers described in Kakoi *et al.* [16] and Han *et al.* [8], respectively. *MC1R*, *MATP*, and *MSTN* were genotyped by PCR-RFLP using *TaqI* α , *MseI*, and *RsaI* restriction enzymes and primers described by Wagner and Reissmann [30], Kakoi *et al.* [16], and Polasik *et al.* [23], respectively. PCR was performed in a 10 μ l reaction volume containing 10 ng genomic DNA,

0.2 μ M primers, 0.25 μ mol/l dNTPs, 2 μ l 5 \times PCR buffer, and 1 U GoTaq DNA polymerase (Promega, Madison, WI, U.S.A.) under the following conditions: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 30–60 sec, annealing at 55–65°C for 30–60 sec, and elongation at 72°C for 30–40 sec; and then final extension at 72°C for 10 min. For PCR-RFLP, the PCR products were digested with the restriction enzyme, and the digested fragments were electrophoresed in an agarose gel. To confirm the accuracy of the genotyping results of PCR-RFLP, the amplified fragments from at least two samples for each genotype of the genes were directly sequenced using the dideoxy method.

Results

Variation in mitochondrial DNA haplotypes

In the present study, we determined mtDNA haplotypes based on the 247-bp sequence of the D-loop region of 22 Kushum horses. As shown in Table 1, 10 haplotypes (KT1–10) with 19 segregated sites were observed in these horses. We excluded four strong mutational hotspots at nucleotide positions 15585, 15597, 15604, and 15650 from the mtDNA haplotype analysis, as described by Cieslak *et al.* [3]. The obtained genotypic diversity, nucleotide diversity, and average nucleotide difference were 0.922 ± 0.034 , 0.02365 ± 0.00239 , and 5.818, respectively.

We also constructed a phylogenetic tree with 97 horse mtDNA haplotypes, including 10 Kushum horse haplotypes and 87 haplotypes reported by Cieslak *et al.* [3], using the neighbor-joining method (Fig. 2) to estimate the phylogenetic relationships of Kushum horse haplotypes with

previously reported haplotypes. Among the 10 haplotypes, 7 haplotypes, namely KT1, 2, 3, 4, 7, 8, and 10, were the same as previously reported haplotypes D2, A, B1, K3, K2b, X2, and X3c1, respectively. These haplotypes are known to be ancestral haplotypes that have survived in modern breeds of horse and are widespread among various primitive breeds of the Eurasian continent [3]. The remaining 3 haplotypes, namely KT5, 6, and 9, were not included in the data set of the 87 previously reported haplotypes [3] but were reported to be present in some primitive breeds or native horse populations in the Eurasian continent [12, 18]. Based on the nucleotide sequences of the D-loop regions of 1,754 modern and 207 ancestral horses, Cieslak *et al.* [3] classified the 87 observed horse mtDNA haplotypes into 17 haplogroups. The 10 haplotypes of the Kushum horses fell into 8 (A, B, D, K, K3, X2, X3, and X7) of the 17 haplogroups, and the distribution of these haplogroups in the Kushum horses was essentially similar to that in primitive horse breeds in Central Asia, including the Akhal-Teke, Caspian, and Vyatskaya [3].

Variation in Y-chromosomal haplotype

We investigated four SNPs (rAX, rW, rA, and rD) in the male-specific euchromatic region of the Y chromosome in seven male Kushum horses. As shown in Table 2, we could classify the Y-chromosomal haplotypes into five groups by the genotypes of these four SNPs [6, 31]. One group was composed of haplotypes outside the crown group defined by Wallner *et al.* [31] (we tentatively named this group HG1), which is mainly observed in Asian local breeds as well as in northern European primitive breeds, including Icelandic horses. The remaining four groups fell inside the

Table 1. Haplotypes of the mtDNA D-loop region observed in Kushum horses

																			Kazakhstan haplotype ^b	Numbers of horses	Haplotypes in Cieslak <i>et al.</i> (2010)
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5			
4	4	4	5	5	5	6	6	6	6	6	6	6	6	7	7	7	7	7			
9	9	9	3	4	9	0	0	1	1	3	4	5	6	6	0	2	2	4			
4	5	6	4	2	8	2	3	6	7	5	9	9	6	7	3	0	6	0			
T	T	A	C	C	T	C	T	A	T	C	A	T	G	A	T	G	G	A			
.	C			
.	C	.	.	.	T	.	.	C	.	.	C	.	.	.	A	.	.	.			
.	C	.	.	.	T	G	C	A	.	.	.			
.	C	.	.	.	T	.	.	C	.	.	C	.	.	C	A	.	.	.			
.	C	.	.	.	T	C	A	A	G	.			
C	C	G	T	.	.	T	C	.	.	.	G	.	.	.	A	.	.	.			
.	C	.	.	.	T	G	.	.	.	C	A	.	G			
.	C	.	.	T	.	T	.	.	.	T	.	.	A	.	C	A	.	.			

^aReference sequence. ^bThe nucleotide sequences of haplotypes KT1–10 were deposited in the DDBJ, EMBL–Bank, and GenBank databases under the following accession numbers: KT1, LC566570; KT2, LC566571; KT3, LC566572; KT4, LC566573; KT5, LC566574; KT6, LC566575; KT7, LC566576; KT8, LC566577, LC566578; KT9, LC566579; KT10, LC566580.

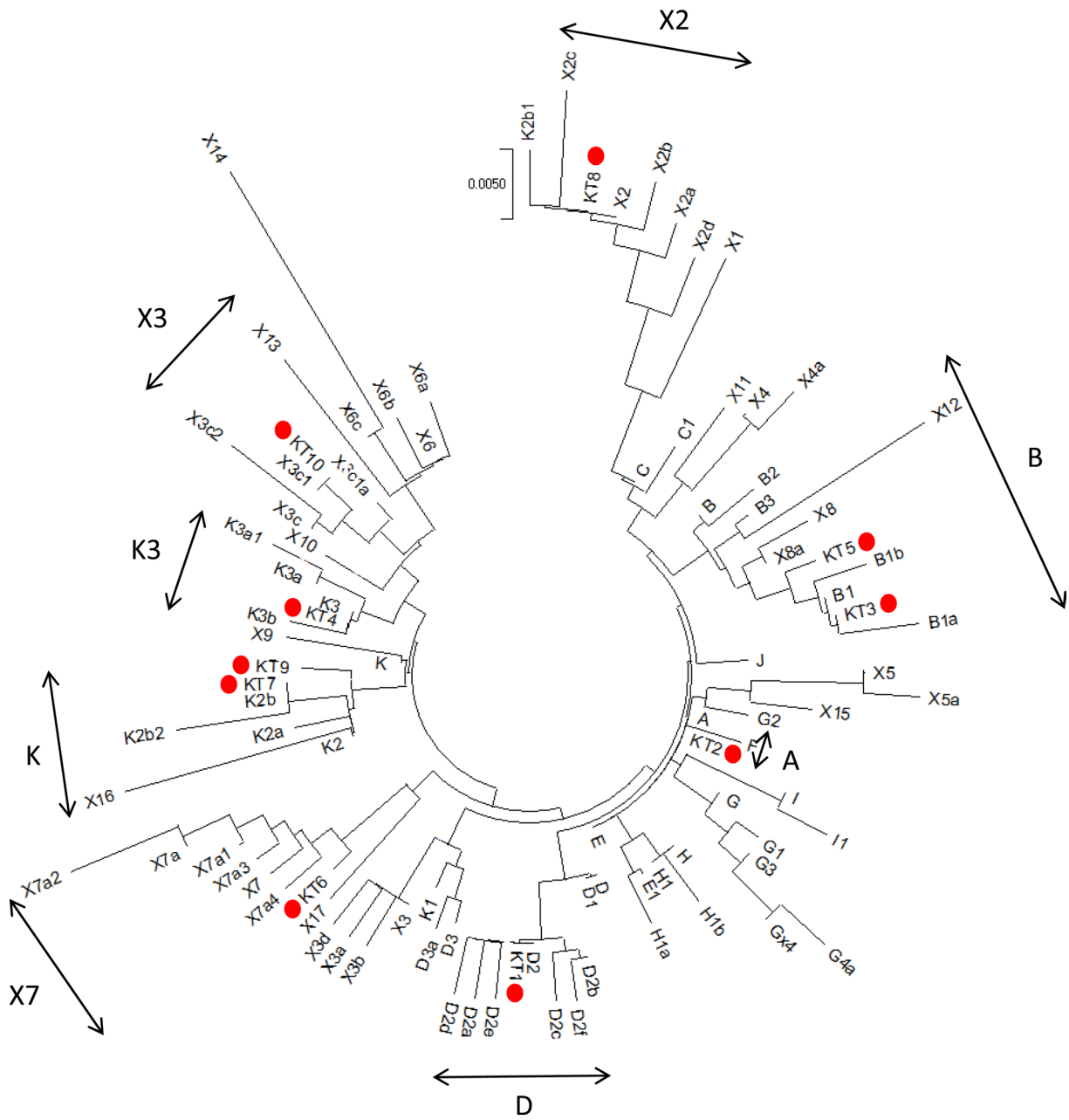


Fig. 2. Neighbor-joining-based phylogenetic tree constructed using the 247-bp sequence of mtDNA D-loop region (15494 to 15740) of 97 haplotypes, including 10 Kushum horse haplotypes (indicated by red circles) and 87 haplotypes reported by Cieslak *et al.* [3].

Table 2. Haplotypes of the Y chromosome observed in Kushum horses

Group of haplotypes	Y-chromosomal markers				Number of horses
	rAX	rA	rW	rD	
HG1	T	T	G	T	0
HG2	C	A	G	T	5
HG3	C	A	G	-	0
HG4	C	T	A	T	2
HG5	C	T	G	T	0

crown group. We tentatively named these groups HG2, HG3, HG4, and HG5, which were composed of haplotypes mainly observed in European and American warmblood breeds, English Thoroughbreds and some of European and American warmblood breeds, Arabian horse-influenced breeds and draft breeds, and some other local breeds, such as the Lipizzan, respectively. The genotyping results of the four SNPs in Y-chromosome indicated that five of the seven male Kushum horses possessed a haplotype that belonged to HG2 and that the remaining two possessed a haplotype that belonged to HG4 (Table 2).

Variation in genes associated with coat color and locomotion traits

In the present study, we also investigated the genotypes of genes associated with coat color (*ASIP*, *MATP*, and *MC1R*) and locomotion traits (*MSTN* and *DMRT3*) of the 22 Kushum horses (Table 3). Regarding coat color-related genes, the genotyping results indicated that all 22 horses carried the wild-type *C* allele of *MATP* associated with a normal coat color and no mutant *C^{cr}* alleles associated with a dilute coat color, suggesting that *MATP* is fixed or nearly fixed for the *C* allele in the population of Kushum horses. For *ASIP* and *MC1R*, both wild-type *A* and *E* alleles and mutant *a* and *e* alleles, respectively, were observed in the 22 horses, in which wildtype alleles were relatively abundant.

Regarding locomotion traits, both *T* and *C* alleles of *MSTN* and *C* and *A* alleles of *DMRT3* were observed in the 22 horses, and *T* allele of *MSTN* associated with high endurance stamina and *C* allele of *DMRT3* associated with normal gait showed higher frequencies in these horses (Table 3).

Discussion

The present findings for the mtDNA haplotypes indicate high genetic diversity and a unique genetic composition of the maternal lineage of Kushum horses. The high genetic diversity of Kushum horses suggested that the founders of this breed were not from limited maternal lineages. Since the Kushum breed was established by crossbreeding between mares of Kazakhstan local horses and stallions of exotic breeds, the mtDNA haplotypes of Kushum horses must have originated from the population of local horses in the early 20th century. Therefore, the high diversity of mtDNA haplotypes in Kushum horses likely reflects the high genetic diversity of the maternal lineage of the local horse population in Central Asia.

Since Kushum horses have been established through three-way crosses of mares of Kazakhstan local horses with stallions of Thoroughbred, Trotter, and Don breeds, the Y-chromosomal haplotypes of Kushum horses must have originated from these exotic breeds. According to Wallner *et al.* [31], most Thoroughbred horses possess a haplotype that belongs to HG3, and Standardbred Trotter horses possess a haplotype that belongs to HG2. While no information about the Y-chromosomal haplotype is available for Don horses, this breed is genetically influenced by Arabian horses [4]. Therefore, the HG2 haplotype of Kushum horses likely originated from the Trotter, and the HG4 haplotype likely originated from the Don, whereas no evidence for the contribution of Thoroughbreds to the Y chromosome of Kushum horses was observed in the horses analyzed in this study. However, another possibility is that both HG2 and HG4 originated from the Don, because the Don breed is also known to have been influenced by the Akhal-Teke [4], which mainly possesses haplotypes belong to HG2 [31], and

Table 3. Genotype distributions and allele frequencies of genes associated with coat color and locomotion traits

Genes	Genotype distributions			Alleles frequencies		χ^2 values for HWE test
	<i>AA</i>	<i>Aa</i>	<i>aa</i>	<i>A</i>	<i>a</i>	
<i>ASIP</i>	12	10	0	0.77	0.23	1.9
<i>MATP</i>	<i>CC</i>	<i>CC^{Cr}</i>	<i>CC^{Cr}</i>	<i>C</i>	<i>C^{Cr}</i>	NA
	22	0	0	1	0	
<i>MC1R</i>	<i>EE</i>	<i>Ee</i>	<i>ee</i>	<i>E</i>	<i>e</i>	0.88
	10	11	1	0.70	0.30	
<i>MSTN</i>	<i>TT</i>	<i>TC</i>	<i>CC</i>	<i>T</i>	<i>C</i>	0.22
	18	4	0	0.91	0.09	
<i>DMRT3</i>	<i>CC</i>	<i>CA</i>	<i>AA</i>	<i>C</i>	<i>A</i>	1.14
	17	4	1	0.86	0.14	

HWE test, Hardy–Weinberg equilibrium test. NA, Not applicable.

Don stallions were used as terminal sires in the three-way cross [5], which dominantly contributed to the introgression of the Y chromosome to offspring.

The coat colors estimated from the genotypes of *ASIP*, *MATP*, and *MC1R* genes were concordant with the observed coat colors, with a few exceptions that might have been caused by misjudgment of coat color upon observation. The allele frequencies of coat color-related genes were essentially consistent with the coat color variation of the Kushum horses, namely mainly bay and chestnut and occasionally black [5]. During field research in Kazakhstan, we have observed various coat colors, including buckskin and palomino, in the local native horse herds. Since Kushum horses originated from the Kazakhstan local horses, our findings of the absence of the *C^{cr}* allele of *MATP* may suggest that the coat colors of Kushum horses have been selected to exclude colors other than the basic coat colors, bay, chestnut, and black, during selective breeding.

The horses with the *TT* genotype in *MSTN* gene were reported to show high endurance stamina in long-distance races, while those with the *CC* genotype were reported to show high speeds in short-distance races [11]. Since the initial goal of breeding of Kushum horses was mainly for military use, endurance for long-distance locomotion might have been essential for this breed. While the *T* allele is the major allele in many horse breeds, the relatively high frequency of the *T* allele of *MSTN* is concordant with such a demand for the Kushum horses.

Horses homozygous for the nonsense mutation (*A* allele) of *DMRT3* can perform ambling gaits, which are characterized by symmetrical lateral movements of the footfall pattern [2, 24]. We found the presence of *A* allele in Kushum horses in the present study, while frequency are lower than *C* allele. Since the frequencies of *A* alleles in most horse breeds are generally very low, except in those with the ability to perform an ambling gait [24], the observed frequency of *A* allele (0.14) in Kushum horse is relatively high. While we have no information on the ability of Kushum horses to perform an ambling gait, one of the initial purposes of the cross with the Trotter during breeding was to improve the gait of Kushum horses [5]. Therefore, the relatively high frequency of the *A* allele might be the result of the introgression of this allele from Trotter stallions, which are known to perform an ambling gait and show a high frequency of the *A* allele [14, 24]. Moreover, gaited horses may have been selected at the early stages of breeding to improve their locomotion traits as military horses, although such a selection may have no longer been performed in later stages of breeding, since these horses were mainly used for milk and meat production subsequently. Thus, the observed frequency of the *A* allele of *DMRT3* might be a trace of such a breeding history.

The breeding of Kushum horses started in 1931, just before World War II, in a major horse-producing region of the former Soviet Union, Kazakhstan. At that time, producing numerous military horses with high endurance performance, good locomotion traits, and strong adaptability to cold climates might have been required to prepare for a possible war on the European continent. Presumably, to meet such military demands, Kushum horses were bred through systematic breeding programs using mares of local horses and stallions of exotic breeds under strong selection pressure for the traits desired in military horses. The allele frequencies of genes associated with locomotion traits observed in the present study might reflect these historical situations. Furthermore, the observed compositions of mtDNA and Y-chromosomal haplotypes are concordant with the documented maternal and paternal origins of this breed. The results of the present study demonstrate that we can obtain useful information for better understanding the origin and breeding history of horse breeds using molecular markers.

Acknowledgments

We would like to thank all the members of the Society for Researchers on Native Livestock who participated in the field research on native livestock in Central Asian countries. This work was supported by a grant from the Japan Society for the Promotion of Science (KAKENHI).

References

1. Achilli, A., Olivieri, A., Soares, P., Lancioni, H., Hooshiar Kashani, B., Perego, U.A., Nergadze, S.G., Carossa, V., Santagostino, M., Capomaccio, S., Felicetti, M., Al-Achkar, W., Penedo, M.C., Verini-Supplizi, A., Houshmand, M., Woodward, S.R., Semino, O., Silvestrelli, M., Giullotto, E., Pereira, L., Bandelt, H.J., and Torroni, A. 2012. Mitochondrial genomes from modern horses reveal the major haplogroups that underwent domestication. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 2449–2454. [[Medline](#)] [[CrossRef](#)]
2. Andersson, L.S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C.J., Patra, K., Arnason, T., Wellbring, L., Hjälm, G., Imsland, F., Petersen, J.L., McCue, M.E., Mickelson, J.R., Cothran, G., Ahituv, N., Roepstorff, L., Mikko, S., Vallstedt, A., Lindgren, G., Andersson, L., and Kullander, K. 2012. Mutations in *DMRT3* affect locomotion in horses and spinal circuit function in mice. *Nature* **488**: 642–646. [[Medline](#)] [[CrossRef](#)]
3. Cieslak, M., Pruvost, M., Benecke, N., Hofreiter, M., Morales, A., Reissmann, M., and Ludwig, A. 2010. Origin and history of mitochondrial DNA lineages in domestic horses. *PLoS One* **5**: e15311. [[Medline](#)] [[CrossRef](#)]

4. Dmitriez, N., and Ernst, L. 1989. DON (Donskaya). pp. 283–285. *In: Animal Genetics Resources of the USSR* (Dmitriez, N., and Ernst, L. eds.), Food and Agriculture Organization of the United Nations, Roma.
5. Dmitriez, N., and Ernst, L. 1989. KUSHUM (Kushums-kaya). pp. 330–331. *In: Animal Genetics Resources of the USSR* (Dmitriez, N., and Ernst, L. eds.), Food and Agriculture Organization of the United Nations, Roma.
6. Felkel, S., Vogl, C., Rigler, D., Jagannathan, V., Leeb, T., Fries, R., Neuditschko, M., Rieder, S., Velie, B., Lindgren, G., Rubin, C.J., Schlötterer, C., Rattei, T., Brem, G., and Wallner, B. 2018. Asian horses deepen the MSY phylogeny. *Anim. Genet.* **49**: 90–93. [[Medline](#)] [[CrossRef](#)]
7. Gurgul, A., Jasielczuk, I., Semik-Gurgul, E., Pawlina-Tyszko, K., Stefaniuk-Szmukier, M., Szmatoła, T., Polak, G., Tomczyk-Wrona, I., and Bugno-Poniewierska, M. 2019. A genome-wide scan for diversifying selection signatures in selected horse breeds. *PLoS One* **14**: e0210751. [[Medline](#)] [[CrossRef](#)]
8. Han, H., Zeng, L., Dang, R., Lan, X., Chen, H., and Lei, C. 2015. The DMRT3 gene mutation in Chinese horse breeds. *Anim. Genet.* **46**: 341–342. [[Medline](#)] [[CrossRef](#)]
9. Han, H., Zhang, Q., Gao, K., Yue, X., Zhang, T., Dang, R., Lan, X., Chen, H., and Lei, C. 2015. Y-single nucleotide polymorphisms diversity in Chinese indigenous horse. *Asian-Australas. J. Anim. Sci.* **28**: 1066–1074. [[Medline](#)] [[CrossRef](#)]
10. Hill, E.W., Bradley, D.G., Al-Barody, M., Ertugrul, O., Splan, R.K., Zakharov, I., and Cunningham, E.P. 2002. History and integrity of thoroughbred dam lines revealed in equine mtDNA variation. *Anim. Genet.* **33**: 287–294. [[Medline](#)] [[CrossRef](#)]
11. Hill, E.W., Gu, J., Eivers, S.S., Fonseca, R.G., McGivney, B.A., Govindarajan, P., Orr, N., Katz, L.M., and MacHugh, D.E. 2010. A sequence polymorphism in MSTN predicts sprinting ability and racing stamina in thoroughbred horses. *PLoS One* **5**: e8645. [[Medline](#)] [[CrossRef](#)]
12. Hristov, P., Yordanov, G., Ivanova, A., Mitkov, I., Sirakova, D., Mehandzyiski, I., and Radoslavov, G. 2017. Mitochondrial diversity in mountain horse population from the South-Eastern Europe. *Mitochondrial DNA A. DNA Mapp. Seq. Anal.* **28**: 787–792. [[Medline](#)]
13. Jansen, T., Forster, P., Levine, M.A., Oelke, H., Hurler, M., Renfrew, C., Weber, J., and Olek, K. 2002. Mitochondrial DNA and the origins of the domestic horse. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 10905–10910. [[Medline](#)] [[CrossRef](#)]
14. Jäderkvist, K., Andersson, L.S., Johansson, A.M., Árnason, T., Mikko, S., Eriksson, S., Andersson, L., and Lindgren, G. 2014. The DMRT3 ‘Gait keeper’ mutation affects performance of Nordic and Standardbred trotters. *J. Anim. Sci.* **92**: 4279–4286. [[Medline](#)] [[CrossRef](#)]
15. Kakoi, H., Kikuchi, M., Tozaki, T., Hirota, K.I., Nagata, S.I., Hobo, S., and Takasu, M. 2018. Distribution of Y chromosomal haplotypes in Japanese native horse populations. *J. Equine Sci.* **29**: 39–42. [[Medline](#)] [[CrossRef](#)]
16. Kakoi, H., Tozaki, T., Nagata, S., Gawahara, H., and Kijima-Suda, I. 2009. Development of a method for simultaneously genotyping multiple horse coat colour loci and genetic investigation of basic colour variation in Thoroughbred and Misaki horses in Japan. *J. Anim. Breed. Genet.* **126**: 425–431. [[Medline](#)] [[CrossRef](#)]
17. Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. [[Medline](#)] [[CrossRef](#)]
18. Lei, C.Z., Su, R., Bower, M.A., Edwards, C.J., Wang, X.B., Weining, S., Liu, L., Xie, W.M., Li, F., Liu, R.Y., Zhang, Y.S., Zhang, C.M., and Chen, H. 2009. Multiple maternal origins of native modern and ancient horse populations in China. *Anim. Genet.* **40**: 933–944. [[Medline](#)] [[CrossRef](#)]
19. Librado, P., and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451–1452. [[Medline](#)] [[CrossRef](#)]
20. Mariat, D., Taourit, S., and Guérin, G. 2003. A mutation in the MATP gene causes the cream coat colour in the horse. *Genet. Sel. Evol.* **35**: 119–133. [[Medline](#)] [[CrossRef](#)]
21. Marklund, L., Moller, M.J., Sandberg, K., and Andersson, L. 1996. A missense mutation in the gene for melanocyte-stimulating hormone receptor (*MC1R*) is associated with the chestnut coat color in horses. *Mamm. Genome* **7**: 895–899. [[Medline](#)] [[CrossRef](#)]
22. McGahern, A., Bower, M.A., Edwards, C.J., Brophy, P.O., Sulimova, G., Zakharov, I., Vizuete-Forster, M., Levine, M., Li, S., MacHugh, D.E., and Hill, E.W. 2006. Evidence for biogeographic patterning of mitochondrial DNA sequences in Eastern horse populations. *Anim. Genet.* **37**: 494–497. [[Medline](#)] [[CrossRef](#)]
23. Polasik, D., Pikula, R., Gawlik, J., Ochman, J., and Terman, A. 2015. Analysis of the myostatin gene (*MSTN*) polymorphism in four breeds of horses. *Piscaria Zootech.* **320**: 81–86.
24. Promerová, M., Andersson, L.S., Juras, R., Penedo, M.C., Reissmann, M., Tozaki, T., Bellone, R., Dunner, S., Hořín, P., Imsland, F., Imsland, P., Mikko, S., Modrý, D., Roed, K.H., Schwochow, D., Vega-Pla, J.L., Mehrabani-Yeganeh, H., Yousefi-Mashouf, N., G Cothran, E., Lindgren, G., and Andersson, L. 2014. Worldwide frequency distribution of the ‘Gait keeper’ mutation in the DMRT3 gene. *Anim. Genet.* **45**: 274–282. [[Medline](#)] [[CrossRef](#)]
25. Rieder, S., Taourit, S., Mariat, D., Langlois, B., and Guérin, G. 2001. Mutations in the agouti (*ASIP*), the extension (*MC1R*), and the brown (*TYRP1*) loci and their association to coat color phenotypes in horses (*Equus caballus*). *Mamm. Genome* **12**: 450–455. [[Medline](#)] [[CrossRef](#)]
26. Signer-Hasler, H., Flury, C., Haase, B., Burger, D., Simianer, H., Leeb, T., and Rieder, S. 2012. A genome-wide association study reveals loci influencing height and other conformation traits in horses. *PLoS One* **7**: e37282. [[Med-](#)

- line] [CrossRef]
27. Tozaki, T., Miyake, T., Kakoi, H., Gawahara, H., Sugita, S., Hasegawa, T., Ishida, N., Hirota, K., and Nakano, Y. 2010. A genome-wide association study for racing performances in Thoroughbreds clarifies a candidate region near the MSTN gene. *Anim. Genet.* **41**(Suppl 2): 28–35. [Medline] [CrossRef]
 28. Tozaki, T., Kikuchi, M., Kakoi, H., Hirota, K.I., and Nagata, S.I. 2017. A genome-wide association study for body weight in Japanese Thoroughbred racehorses clarifies candidate regions on chromosomes 3, 9, 15, and 18. *J. Equine Sci.* **28**: 127–134. [Medline] [CrossRef]
 29. Vilà, C., Leonard, J.A., Gotherstrom, A., Marklund, S., Sandberg, K., Liden, K., Wayne, R.K., and Ellegren, H. 2001. Widespread origins of domestic horse lineages. *Science* **291**: 474–477. [Medline] [CrossRef]
 30. Wagner, H.J., and Reissmann, M. 2000. New polymorphism detected in the horse MC1R gene. *Anim. Genet.* **31**: 289–290. [Medline] [CrossRef]
 31. Wallner, B., Palmieri, N., Vogl, C., Rigler, D., Bozlak, E., Druml, T., Jagannathan, V., Leeb, T., Fries, R., Tetens, J., Thaller, G., Metzger, J., Distl, O., Lindgren, G., Rubin, C.J., Andersson, L., Schaefer, R., McCue, M., Neuditschko, M., Rieder, S., Schlotterer, C., and Brem, G. 2017. Y chromosome uncovers the recent oriental origin of modern stallions. *Curr. Biol.* **27**: 2029–2035.e5. [Medline] [CrossRef]
 32. Wallner, B., Vogl, C., Shukla, P., Burgstaller, J.P., Druml, T., and Brem, G. 2013. Identification of genetic variation on the horse Y chromosome and the tracing of male founder lineages in modern breeds. *PLoS One* **8**: e60015. [Medline] [CrossRef]