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FULL PAPER

Single nucleotide polymorphisms of *Kit* gene in Chinese indigenous horses

Haoyuan Han, Chunchun Mao, Ningbo Chen, Xianyong Lan, Hong Chen, Chuzhao Lei*) and Ruihua Dang*)

College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China

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Abstract

***Kit* gene is a genetic determinant of horse white coat color which has been a highly valued trait in horses for at least 2,000 years. Single nucleotide polymorphisms (SNPs) in *Kit* are of importance due to their strong associations with melanoblast survival during embryonic development. In this study, a mutation analysis of all 21 *Kit* exons in 14 Chinese domestic horse breeds revealed six SNPs (g.91214T>G, g.143245T>G, g.164297C>T, g.170189C>T, g.171356C>G, and g.171471G>A), which located in 5'-UTR region, intron 6, exon 15, exon 20, intron 20, and exon 21 of the equine *Kit* gene, respectively. Subsequently, these six SNPs loci were genotyped in 632 Chinese horses by PCR-RFLP or direct sequencing. The six SNPs together defined 18 haplotypes, demonstrating abundant haplotype diversities in Chinese horses. All the mutant alleles and haplotypes were shared among different breeds. But fewer mutations were detected in horses from China than that from abroad, indicating that Chinese horses belong to a more ancient genetic pool. This study will provide fundamental genetic information for evaluating the genetic diversity of *Kit* gene in Chinese indigenous horse breeds.**

Key Words: Chinese horses; *Kit*; SNPs; white coat color

Introduction

White spotting patterns and the flashy spotted coats are valued in the horse for their aesthetic quality. White coat horses lack pigment in both the hair and the skin. The effect on depigmentation is the most visible phenotypic change and can range from tiny white spots to a completely white coat. Thus far, four depigmentation phenotypes including dominant white (W)[6], roan (Rn)[16], sabino-1 (Sb)[2], and tobiano (To)[3] have been observed and independently mapped to a region on equine chromosome 3 (ECA 3) harboring the equine *v-Kit Hardy-Zuckerman 4 feline sarcoma*

*Corresponding author: Ruihua Dang, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
Phone: 15388627637. E-mail: dangruihua@nwsuaf.edu.cn
Chuzhao Lei, College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China
Phone: +86-29-87092004. Fax: +86-29-87092164. E-mail: leichuzhao1118@126.com
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viral oncogene homolog (*Kit*) gene by linkage analysis[17].

Kit belongs to the receptor tyrosine kinase (*PTK*) gene family proposed to have evolved from a common ancestral gene by duplications. Several studies on human, mice, and pigs observed pleiotropic effects of *Kit* mutations. Kit signaling displays a key role for the proliferation and differentiation of hematopoietic cells, germ cells, mast cells, and interstitial cells of Cajal (ICC)[1,7,12,21]. Thus, *Kit* gene plays an essential role in different aspects of biological process. The equine *Kit* gene spans a genomic region of about 82 kb and comprises 21 exons[6]. The equine *Kit* mRNA (NCBI accession number: AM420315) contains an open reading frame (ORF) of 2,919 bp. It transcribes 972 animo acids encoding a tyrosine kinase receptor which is a transmembrane protein and specifically binds extracellular ligands followed by signal transduction into the cell[20]. This contributes to their important role in the control of melanoblasts proliferation, survival, motility, and differentiation[21].

Kit signaling acts as an essential survival factor for the differentiation of melanoblasts to mature melanocytes, though the process is also under the control of several other factors[11,15,19]. Melanoblasts are derived from either side of the neural crest and start to express *Kit* from the time they leave the neural crest. Then melanocytes distally toward the extremities, and finally enter their final location in the epidermis[18,22]. Thus, loss-of-function mutations in the *Kit* gene lead to reduced migration, proliferation, or survival of melanoblasts in the embryo, resulting in the loss of melanocytes and the subsequent formation of unpigmented skin patches[17,19]. To date, 20 functionally different W alleles of the *Kit* gene have been characterized as causative candidate mutations at the molecular level in horses, with phenotypes ranging from small areas of depigmentation to white over the entire body[2,5–9]. Therefore, searching for candidate mutations is a prerequisite to evaluate and protect diversity of horse coat color.

There are 29 local domestic horse breeds and many populations throughout 14 provinces in Northwestern, Southwestern, Northeastern, and Central China[4]. Chinese indigenous horse can be divided into 5 groups: Mongolia horse, Southwest horse, Yushu horse, Kazakh horse, and Hequ horse based on their history, ecological environment and body size[4]. As well known, Chinese horse played a very significant role in transportation and war and used to be placed in the first position of six domestic animals (cattle, sheep, pig, dog, and chicken) in ancient China. But the number and genetic diversity of Chinese horse is decreasing with the development of society. Thus we proposed to protect the variety resources based on the level of genetic diversity of Chinese horses.

Currently, study on genetic polymorphisms of *Kit* gene in Chinese horses has not been reported. Here, this research was undertaken to study the genetic variations of *Kit* gene in 632 Chinese horses representing 14 breeds[4]. All 21 exons and their flanking regions were screened and six SNPs were detected. Our study will provide basic genetic information of *Kit* mutations in Chinese horses, which is valuable for the protection and preservation of genetic sources of domestic Chinese horse breeds.

Materials and methods

Specimen collection and DNA extraction: A total of 632 blood samples representing 14 Chinese native horse breeds were collected from different regions across China. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Northwest A&F University. The horse samples have already been used in previous studies[23]. Detail information of the horse breeds analyzed in this study was presented

Table 1. *KIT* gene genetic diversity and breeds information in Chinese horses

Breed	Code	No.	h	Hd (SD)	π (SD) %
Lichuan	LCH	41	12	0.793 (0.060)	0.12317 (0.01814)
Debao	DB	47	11	0.812 (0.050)	0.12843 (0.01633)
Baise	BS	40	12	0.879 (0.037)	0.17073 (0.02221)
Guizhou	GZ	66	13	0.708 (0.060)	0.11033 (0.01612)
Yushu	YS	64	17	0.842 (0.041)	0.14269 (0.01422)
Hequ	HQ	39	8	0.704 (0.079)	0.11538 (0.02211)
Chaidamu	CDM	60	12	0.669 (0.067)	0.09586 (0.01556)
Datong	DT	32	10	0.746 (0.080)	0.12046 (0.02376)
Yanqi	YJ	62	11	0.832 (0.039)	0.13317 (0.01364)
Balikun	BLK	33	10	0.786 (0.070)	0.11490 (0.01774)
Kazakh	KZK	22	10	0.835 (0.077)	0.13203 (0.02111)
Guanzhong	GU	29	7	0.956 (0.022)	0.22126 (0.02133)
Ningqiang	NQ	39	11	0.761 (0.072)	0.13563 (0.02129)
Chakouyi	CKY	58	13	0.742 (0.061)	0.12447 (0.01803)
ALL		632	18	0.788 (0.016)	0.13006 (0.00510)

h, haplotypes; Hd, haplotype diversity (standard deviation); π, nucleotide diversity (standard deviation).

in Table 1. Genomic DNA was extracted from jugular blood using Genomic DNA isolation kit (Sangon, Shanghai, China) according to the manufacturer's instructions.

PCR amplification and genotyping by PCR-RFLP or direct sequencing: The DNA fragments of 21 exons including flanking regions of the equine *Kit* gene were amplified. Primers were obtained from two studies: exons 1, 4 and 15[10], and the other exons[6]. Seventy DNA pools (each including 10 individuals) were blended and used to identify SNP in equine *Kit* gene by direct sequencing with ABI PRIZM 377 DNA sequencer (Perkin-Elmer) (Shanghai Sangon Biotech Company, Shanghai, China). PCR amplifications were performed in 12.5 μL reactions containing 10 ng genomic DNA, 5 pM each primer, 6.25 μL 2 × PCR Mix buffer (including 1 U Taq DNA polymerase, 2 × PCR buffer, 3 mM $MgCl_2$, and 400 μM dNTPs) (CWBio, China) with the following conditions: 4 min at 95°C, followed by 36 cycles for 30 s at 94°C, 60 s at 55–60°C, 90 s at 72°C, a final extension of 10 min at 72°C, at last storing at 4°C.

PCR-RFLP protocols were designed to genotype and verify the SNPs identified from the horse DNA pools. Three restriction enzymes, *Taq*I, *TRU1*I, and *Hin6*I, were chosen to genotype the SNPs in exon 15, 20, and 21, respectively (Table 2). In the digestion process, 7 μL of PCR products mixed through with 2 μL of 10 × buffer, 1 μL restriction enzymes (TaKaRa Biotechnology), 2 μL 0.1% BSA and then digested at corresponding temperature (Table 2) for 3 h, following the supplier's instructions. digested products were visualized in 3% agarose gel (Table 2). While other SNPs were genotyped by sequencing for the sites failed to find any opportune restriction enzymes.

Data analysis: DNA sequences of PCR products from DNA pools were edited using the DNASTAR 5.0 package (DNAstar, Madison, Wis., USA) and aligned by ClustalX version 2.0. Based on the genotyping information among the analyzed Chinese horse breeds, genotypic frequencies were directly calculated. The haplotypes existed in each breed and the corresponding haplotype frequencies, and Linkage disequilibrium (LD) across the six SNPs were estimated online (http://

Table 2. *KIT* gene polymorphisms in Chinese horse breeds and the PCR-RFLP conditions

SNPs[a]	Position	RFLP Primers	PCR Product Size (bp)	Endonuclease	Digested Product Size	SNP type
g.91214T>G	5'-UTR					
g.143245T>G	Intron 6					
g.164297C>T	Exon 15	F: TCATTCAAACTTGGCAATACTT R: CTCTTGTCTGTCTTGGTGGGTTC	226	*Taq* I	CC 205 bp; CT 205 bp and 226 bp; TT 226 bp	Silent
g.170189C>T	Exon 20					
g.171356C>G	Intron 20	F: TTGCTGGGATGCTGATC R: AAGCCAAGGAGGGAAGG	201	*TRU1* I	CC 201 bp; CG 167 bp and 201 bp; GG 167 bp	Silent
g.171471G>A	Exon 21	F: TCTGAGATGTGTCCCAGCAG R: TCATTCTTGTTGGGGAGACC	429	*Hin6* I	GG 81 bp and 348 bp; GA 81 bp, 348 bp, and 429 bp	p.Ala960Thr

[A]Numbering refers to accession number AM420315.

analysis.bio-x.cn/myAnalysis.php). Haplotype was eliminated when the frequency was lower than 0.03 in all samples. Haplotype diversity and nucleotide diversity for each breed were estimated using the DnaSPv5. A network was constructed to investigate the relationship among haplotypes by Network 4.6.1.3 (Fluxus Technology Ltd., 2012, Kiel, Germany).

Results

All of 632 individuals representing 14 Chinese horse breeds were screened to investigate the genetic diversity of the equine *Kit* gene. Comparison of the sequences we obtained from the equine DNA pools with the reference sequence revealed six polymorphisms (g.91214T>G, g.143245T>G, g.164297C>T, g.170189C>T, g.171356C>G, and g.171471G>A) (Table 3). Three SNPs were found in the exons, among which, we identified a mutation (g.171471G>A) affecting the *Kit* coding sequence, resulting in amino acid changes (p.A960T) and the other two polymorphisms were synonymous mutation (g.164297C>T and g.170189C>T). The other three SNPs were located in the introns and the 5'-UTR region of the equine *Kit* gene.

Three SNPs, g.164297C>T, g.170189C>T, and g.171471G>A, can be genotyped by natural endonuclease restriction sites, while g.91214T>G, g.143245T>G, and g.171356C>G were genotyped by direct sequencing. The mutations g.91214T>G, g.143245T>G, and g.171471G>A only displayed two genotypes (TT and TG, TT and TG, GG and GA, respectively), whereas the remaining three SNP loci each demonstrated three genotypes. Frequencies of each site in 14 Chinese native breeds were presented in Table 3. All the alleles were not restricted to a specific breed, instead, existed in all Chinese horse breeds analyzed in our study. But the wild homologous genotypes were dominant at each locus, accounting for more than 83% in every horse breed (Table 3). We performed linkage disequilibrium (LD) analysis in 14 horse breeds, among which the genotype distributions were in Hardy-Weinberg equilibrium. From the result of the LD analysis, we found that SNP1/SNP3 of Hequ (HQ) and SNP2/SNP5 of Guanzhong (GU) were closely linked loci based on the r^2 and D' values. In contrast, SNP1/SNP3 and SNP5/SNP6 of Baise (BS) ($r^2 = 0.000$, $D' = 0.000$) and SNP2/SNP4 of Guizhou (GZ) ($r^2 = 0.000$, $D' = 0.000$) were suggested to be completely mutual independence (Table 4).

To further analyze the above six variations of the equine *Kit* gene, haplotype analysis were performed by combining them together. Hence, a total of 18 haplotypes (HAP1-HAP18) were identified (Table 5). The network clearly revealed that HAP1 was in the center position and shared by most individuals, which indicated HAP1

Table 3. Genotyping for mutations in the horse *KIT* gene

Breed	No.	g.91214T>G		g.143245T>G		g.164297C>T			g.170189C>T			g.171356C>G			g.171471G>A	
		TT	TG	TT	TG	CC	CT	TT	CC	CT	TT	CC	CG	GG	GG	GA
LCH	41	37 (0.902)	4 (0.098)	37 (0.902)	4 (0.098)	36 (0.878)	4 (0.098)	1 (0.024)	34 (0.829)	6 (0.146)	1 (0.024)	33 (0.805)	7 (0.171)	1 (0.024)	38 (0.927)	3 (0.073)
DB	47	41 (0.872)	6 (0.128)	42 (0.894)	5 (0.106)	37 (0.787)	9 (0.191)	1 (0.021)	41 (0.872)	5 (0.106)	1 (0.021)	41 (0.872)	5 (0.106)	1 (0.021)	42 (0.894)	5 (0.106)
BS	40	32 (0.800)	8 (0.200)	36 (0.900)	4 (0.100)	31 (0.775)	8 (0.200)	1 (0.025)	34 (0.850)	5 (0.125)	1 (0.025)	27 (0.675)	10 (0.250)	3 (0.075)	35 (0.875)	5 (0.125)
GZ	66	56 (0.848)	10 (0.152)	60 (0.909)	6 (0.091)	58 (0.879)	6 (0.091)	2 (0.030)	58 (0.879)	5 (0.076)	3 (0.045)	61 (0.924)	4 (0.061)	1 (0.015)	61 (0.924)	5 (0.076)
YS	64	54 (0.844)	10 (0.156)	58 (0.906)	6 (0.094)	55 (0.859)	9 (0.141)	0.000	50 (0.781)	11 (0.172)	3 (0.047)	53 (0.828)	10 (0.156)	1 (0.016)	54 (0.844)	10 (0.156)
HQ	39	33 (0.846)	6 (0.154)	36 (0.923)	3 (0.077)	37 (0.949)	2 (0.051)	0.000	32 (0.821)	5 (0.128)	2 (0.051)	34 (0.872)	4 (0.103)	1 (0.026)	35 (0.897)	4 (0.103)
CDM	60	53 (0.883)	7 (0.117)	57 (0.950)	3 (0.050)	53 (0.883)	7 (0.117)	0.000	51 (0.850)	7 (0.117)	2 (0.033)	54 (0.900)	6 (0.100)	0.000	56 (0.933)	4 (0.067)
DT	32	28 (0.875)	4 (0.125)	30 (0.938)	2 (0.062)	28 (0.875)	2 (0.062)	2 (0.062)	28 (0.875)	3 (0.094)	1 (0.031)	29 (0.906)	2 (0.062)	1 (0.031)	28 (0.875)	4 (0.125)
YJ	62	51 (0.823)	11 (0.177)	58 (0.935)	4 (0.065)	49 (0.790)	13 (0.210)	0.000	50 (0.806)	10 (0.161)	2 (0.032)	52 (0.839)	8 (0.129)	2 (0.032)	58 (0.935)	4 (0.065)
BLK	33	29 (0.879)	4 (0.121)	31 (0.939)	2 (0.061)	29 (0.879)	4 (0.121)	0.000	29 (0.879)	2 (0.061)	2 (0.061)	27 (0.818)	5 (0.152)	1 (0.030)	31 (0.939)	2 (0.061)
KZK	22	18 (0.818)	4 (0.182)	20 (0.909)	2 (0.091)	19 (0.864)	3 (0.136)	0.000	17 (0.773)	5 (0.227)	0.000	18 (0.818)	4 (0.182)	0.000	20 (0.909)	2 (0.091)
GU	29	21 (0.724)	8 (0.276)	26 (0.897)	3 (0.103)	19 (0.655)	7 (0.241)	3 (0.103)	22 (0.759)	5 (0.172)	2 (0.069)	19 (0.655)	9 (0.310)	1 (0.034)	23 (0.793)	6 (0.207)
NQ	39	30 (0.769)	9 (0.231)	35 (0.897)	4 (0.103)	31 (0.795)	7 (0.179)	1 (0.026)	32 (0.821)	7 (0.179)	0.000	35 (0.897)	3 (0.077)	1 (0.026)	36 (0.923)	3 (0.077)
CKY	58	52 (0.897)	6 (0.103)	54 (0.931)	4 (0.069)	51 (0.879)	6 (0.103)	1 (0.017)	47 (0.810)	9 (0.155)	2 (0.034)	47 (0.810)	8 (0.138)	3 (0.052)	54 (0.931)	4 (0.069)
Total	632	535 (0.847)	97 (0.153)	580 (0.918)	52 (0.082)	533 (0.843)	87 (0.138)	12 (0.019)	525 (0.831)	85 (0.134)	22 (0.035)	530 (0.839)	85 (0.134)	17 (0.027)	571 (0.903)	61 (0.097)

Table 4. The estimated values of linkage equilibrium analysis between six SNPs within *Kit* gene of studied population

SNP	LCH		DB		BS		GZ		YS		HQ		CDM		DT		YJ		BLK		KZK		GU		NQ		CKY	
	r2	D'	r^2	D'	r^2	D'	r^2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'	r2	D'
SNP1/SNP2	0.003	1.000	0.004	1.000	0.072	0.390	0.000	0.018	0.000	0.012	0.003	1.000	0.026	0.250	0.093	0.437	0.003	0.091	0.094	0.440	0.005	1.000	0.002	0.084	0.197	0.689	0.019	0.169
SNP1/SNP3	0.004	1.000	0.069	0.367	0.000	0.000	0.007	1.000	0.045	0.225	0.316	1.000	0.136	0.369	0.007	1.000	0.011	0.991	0.024	0.156	0.029	0.199	0.046	0.997	0.110	0.331	0.001	0.035
SNP1/SNP4	0.073	0.394	0.005	1.000	0.011	1.000	0.008	0.096	0.008	0.121	0.027	0.207	0.028	0.214	0.027	0.186	0.012	0.993	0.006	1.000	0.114	0.382	0.029	0.998	0.060	0.282	0.019	0.210
SNP1/SNP5	0.006	1.000	0.056	0.258	0.031	0.265	0.004	1.000	0.019	0.151	0.007	1.000	0.003	1.000	0.023	0.152	0.010	0.985	0.008	1.000	0.174	0.417	0.000	0.015	0.029	0.236	0.016	0.198
SNP1/SNP6	0.002	0.999	0.255	0.557	0.042	0.266	0.002	0.059	0.005	0.820	0.009	0.120	0.014	0.156	0.004	1.000	0.003	0.091	0.002	1.000	0.005	1.000	0.006	0.092	0.005	1.000	0.002	1.000
SNP2/SNP3	0.011	0.128	0.007	1.000	0.008	1.000	0.045	0.279	0.001	0.032	0.001	1.000	0.002	1.000	0.003	1.000	0.004	1.000	0.002	1.000	0.123	0.434	0.033	0.419	0.055	0.366	0.009	0.135
SNP2/SNP4	0.006	1.000	0.005	1.000	0.017	0.178	0.000	0.000	0.008	1.000	0.005	1.000	0.009	0.188	0.003	1.000	0.004	1.000	0.003	1.000	0.006	1.000	0.010	1.000	0.005	0.091	0.005	1.000
SNP2/SNP5	0.010	0.158	0.005	1.000	0.020	0.305	0.002	1.000	0.017	0.188	0.023	0.220	0.001	1.000	0.002	1.000	0.004	1.000	0.037	0.374	0.005	1.000	0.233	1.000	0.004	1.000	0.041	0.397
SNP2/SNP6	0.054	0.269	0.003	1.000	0.155	0.443	0.013	0.124	0.004	1.000	0.052	0.266	0.001	1.000	0.002	1.000	0.001	1.000	0.001	1.000	0.002	1.000	0.013	0.169	0.002	1.000	0.001	1.000
SNP3/SNP4	0.009	1.000	0.011	1.000	0.013	0.967	0.000	0.018	0.011	0.990	0.026	0.357	0.028	0.214	0.009	1.000	0.010	0.839	0.006	1.000	0.009	1.000	0.000	0.013	0.060	0.282	0.026	0.210
SNP3/SNP5	0.003	0.064	0.011	1.000	0.003	0.075	0.006	0.098	0.006	0.910	0.002	1.000	0.003	0.060	0.019	0.172	0.011	0.107	0.001	0.052	0.007	1.000	0.000	0.005	0.005	0.096	0.010	1.000
SNP3/SNP6	0.003	1.000	0.022	0.229	0.010	1.000	0.003	1.000	0.003	0.664	0.001	1.000	0.002	1.000	0.007	1.000	0.001	0.052	0.002	1.000	0.003	1.000	0.001	0.045	0.005	1.000	0.003	1.000
SNP4/SNP5	0.000	0.017	0.000	0.086	0.000	0.023	0.004	1.000	0.000	0.156	0.002	0.058	0.000	0.198	0.027	0.186	0.001	0.036	0.012	1.000	0.114	0.382	0.043	1.000	0.007	1.000	0.035	0.194
SNP4/SNP6	0.004	1.000	0.003	0.063	0.071	0.319	0.007	0.127	0.001	0.247	0.068	0.405	0.003	1.000	0.011	0.119	0.004	1.000	0.003	1.000	0.006	1.000	0.000	0.014	0.004	1.000	0.007	0.155
SNP5/SNP6	0.007	0.152	0.005	1.000	0.000	0.000	0.013	0.124	0.005	0.082	0.023	0.187	0.002	1.000	0.004	1.000	0.156	0.707	0.004	1.000	0.005	1.000	0.004	0.390	0.048	0.288	0.005	1.000

Table 5. The haplotype frequencies for six SNPs in *KIT* gene in studied populations

Haplotype	SNPs						Frequency in population												
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	LCH (n = 41)	DB (n = 47)	BS (n = 40)	GZ (n = 66)	YS (n = 64)	HQ (n = 39)	CDM (n = 60)	DT (n = 32)	YJ (n = 62)	BLK (n = 34)	KZK (n = 22)	GU (n = 29)	NQ (n = 39)	CKY (n = 58)
HAP1	T	T	C	C	C	G	0.678	0.663	0.584	0.716	0.634	0.728	0.760	0.683	0.620	0.676	0.699	0.356	0.712	0.700
HAP2	T	T	C	T	C	G	0.063	0.058	0.032	0.055	0.098	0.078	0.062	0.051	0.096	0.091	0.049	0.138	0.028	0.039
HAP3	T	T	C	C	G	G	0.062	0.047	0.104	0.031	0.034	0.052	0.038	0.031	0.034	0.080	0.025	0.121	0.027	0.064
HAP4	T	T	C	C	C	A	0.014	0.012	0.014	0.009	0.044	0.013	0.026	0.051	—	0.030	0.045	0.023	0.026	0.026
HAP5	T	T	T	C	C	G	0.050	0.087	0.071	0.053	0.034	—	0.027	0.078	0.076	0.036	0.024	0.155	0.041	0.036
HAP6	G	T	C	C	C	G	0.027	—	0.044	0.049	0.019	0.038	0.018	0.032	0.072	0.032	—	0.092	0.029	0.020
HAP7	T	G	C	C	C	G	0.013	0.053	0.013	0.016	0.025	0.014	0.009	0.016	0.027	—	0.024	—	0.013	0.009
HAP8	G	G	C	C	C	G	—	—	—	0.006	—	—	0.008	0.015	0.005	0.015	—	—	—	—
HAP9	T	T	T	C	G	G	0.012	—	0.027	0.008	0.006	—	0.007	0.016	0.017	0.011	—	—	—	—
HAP10	T	T	C	T	G	G	0.012	0.006	0.022	—	0.003	—	0.005	—	0.013	—	0.021	—	—	0.025
HAP11	G	T	C	C	C	A	—	0.012	—	0.007	0.007	—	0.008	—	—	—	—	0.028	—	—
HAP12	T	T	C	T	C	A	—	0.010	0.012	0.008	0.008	—	—	0.012	—	—	—	—	—	0.009
HAP13	G	T	C	T	C	G	0.022	—	—	0.014	0.008	—	—	—	—	—	0.024	—	0.013	0.008
HAP14	T	T	C	C	G	A	0.011	—	—	0.007	0.015	—	—	—	0.024	—	—	—	0.013	—
HAP15	G	T	C	C	G	G	—	0.021	—	—	0.008	—	—	—	0.004	—	0.024	—	0.012	0.007
HAP16	T	T	T	T	C	G	—	—	0.009	—	0.003	—	—	—	—	—	—	—	0.025	0.024
HAP17	G	T	T	C	C	G	—	0.011	0.010	—	0.015	0.014	0.008	—	—	0.013	0.023	—	—	—
HAP18	T	G	C	C	G	G	0.012	—	—	—	0.014	0.012	—	—	—	0.015	—	—	—	0.017

Hap, Haplotype; SNP, Single Nucleotide Polymorphism.
SNP1, g.91214T>G; SNP2, g.143245T>G; SNP3, g.164297C>T; SNP4, g.170189C>T; SNP5, g.171356C>G; SNP6, g.171471G>A;
CDM, Chaidamu; CKY, Chakouyi; DT, Datong; YJ, Yanji; BS, Baise; DB, Debao pony; GU, Guanzhong; GZ, Guizhou; NQ, Ningqiang; BLK, Balikun; KZK, Kazakh; YS, Yushu; HQ, Hequ; LCH, Lichuan.

maybe an ancestral haplotype. All haplotypes were present in more than four breeds, of which three (HAP1-3) existed in every breed (Table 4). Yushu (YS) breed possessed the highest haplotype number (17), while GU had the lowest haplotype number (7) (Table 5). But only 35.6% samples of GU displayed wild homologous haplotype (HAP1), so the GU had the highest haplotype diversity (0.956) and nucleotide diversity (0.22126). More than half of the individuals of other 13 breeds possessed HAP1, among which, Chaidamu (CDM) had the highest frequency of HAP1 (76%). And CDM had the lowest haplotype diversity (0.669) and nucleotide diversity (0.09586).

Discussion

We compared six SNPs which were identified in Chinese horses with the reported *Kit* mutations, finding that all the six *Kit* gene mutations were also detected in horses from other countries, where these six SNPs substitutions were not found exclusively in white or white-spotted horses but also in solid-colored horses[6,7]. So they were speculated as be non-candidate alleles for horse white coat. All the 43 non-candidate *Kit* polymorphisms were segregated in at least two distinct horse populations[2,5–9], indicating that they spread into different horse populations by the ongoing admixture, which was typical for many modern horse breeds. But only six *Kit* gene SNPs were identified in the local Chinese horses, presenting a lower *Kit* gene diversity of horses from China than that from abroad. The evolution evidence showed that the coat color variance arose rapidly during domestication as a result of human selections[5]. At the beginning, only bay color was present. A rapid and substantial increase in the number of coat colorations was found beginning in the 5000 B.P.. Thus we suggested that the *Kit* gene of Chinese horses were from a more ancient gene pool.

Until now, a total of 63 mutations were reported in equine *Kit* gene[2,5–9], showing a remarkable allelic diversity. But we recognized that the frequency for each mutation was very low not even exceeding 0.2 in our study, suggesting that the observed allelic diversity did not necessarily implicate a particularly high mutation rate. It is conceivable that the striking coat color phenotypes of the horses and the dominant inheritance increase the chances that spontaneous mutations in this gene are recognized in the first place[7].

Up to now, 20 alleles were proved to be responsible for horse white or spotted phenotypes[2,5–9]. These 20 candidate causative mutations were absent from 632 unknown coat color phenotype Chinese horses. Several possible reasons were proposed for this phenomenon. Firstly, the white phenotype associated polymorphisms were detected exclusively in some special white horse families. In the previous studies, the 20 proposed candidate causative mutations they found segregated only within the 20 respective families[2,5–9]. Secondly, many of the described W alleles arose during the last 10 years. One example for such a scenario is the W8 allele, which was observed in a single mottled Icelandic horse as the founder animal for this mutation[7]. Without cross breeding with other families or breeds, these W alleles were certainly limited in one white horse families. Lastly, the samples we analyzed were randomly selected, among which there might be no white individuals[7]. Coat color dilutions or spottings approximately existed in the Bronze Age[14], and mutations responsible for white coat or white spotted depigmentation seemed to appear at that time. In line with the recent origin of these mutation events, no causative variation was found in the Chinese horses, suggesting that none of these horses was white or white spotted. It was speculated that the *Kit* gene of Chinese horses belonged to a more ancient genetic pool, which was consistent with our result of mitochondria DNA study regarding to the horse evolution and domestication[13]. So that it was reasonable that no candidate causative mutations for white coat color

of the equine *Kit* gene were detected in this study.

From the LD analysis result, SNP1/SNP3 of HQ and SNP2/SNP5 of GU were in LD, resulting from incomplete mixture of local breed horses. GU is a cultivated breed and varietal hybridization was adopted to increase the population quality during 1950–1965. Mongolia horse was substantially introduced into the HQ population along with the Mongol attack in Yuan dynasty (AD 1280–1368). During 1983–2005, the HQ stock was decreased by 27.8% because of the horse restrictions policy and its less important role in transportation and agriculture[4]. So less attention was paid to the HQ selection breeding. Based on the analysis of the nucleotide diversity and haplotype diversity of Chinese horse breed, CDM displayed the lowest genetic diversity. It was apprehensible in ways that CDM distributed in the secluded and pastoral area of northwest China, with tough natural environment. In the past decades, CDM played a less important role in transportation and agriculture and suffered from a bottleneck, resulting in a rapid decrease of the population size. By contrast, the GU revealed the highest nucleotide diversity and haplotype diversity which lived in the broad Guanzhong plain, leading to a frequent gene flow among different horse breeds.

In conclusion, our study investigated the genetic variance of *Kit* gene in 632 Chinese horses and found six SNPs in all (g.91214T>G, g.143245T>G, g.164297C>T, g.170189C>T, g.171356C>G, and g.171471G>A), of which, three were located in the coding region of the *Kit* and one caused amino acid change. But the number of mutations was quite smaller than that confirmed abroad and the six loci we determined had no association with horse white coat phenotype. Based on the genetic evolution of *Kit* gene and the coat coloration in horses, we suggested that Chinese horses came from a more ancient gene pool.

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