

# Identification of Y Chromosome Genetic Variations in Chinese Indigenous Horse Breeds

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Y chromosome acts as a single nonrecombining unit that is male specific and in effect haploid, thus ensuring the preservation of mutational events as a single haplotype via male lines. In this study, 6 Y chromosome-specific microsatellites (SSR) were tested for the patrilineal genetic variations of 573 male samples from Chinese domestic horse (30 breeds), Przewalski's horse, and donkey. All the 6 loci appeared as a haplotype block in Przewalski's horse and the domestic donkey. There were notable differences, however, at Y chromosome markers between horse and donkey. There were 2 haplotypes of Eca.YA16 in the domestic horse breeds, Haplotype A (Allele A: 156 bp) and Haplotype B (Allele B: 152 bp). Allele A was the common allele among 30 horse breeds, and Allele B was found in 11 horse breeds. This is the first description of a Y chromosome variant for horses. The 2 haplotypes of Y chromosome discovered in the domestic horse breeds in China could be helpful in unveiling their intricate genetic genealogy.

**Key words:** *haplotype, horse, microsatellite, phylogeography, Y chromosome*

The entire Y chromosome except the pseudoautosomal region is male specific and actually haploid and constitutes a nonrecombinant block. Genealogical DNA tests in human Y chromosome suggested that Y-DNA haplotypes may develop into an important tool for population genetics. In contrast to the exhaustive sequence data on human and mouse, Y chromosome information is fairly scarce for other mammals (Hurles and Jobling 2001).

For equids, mitochondrial DNA and autosomal microsatellites have been applied in intragenus phylogenetic

relationship determination (Oakenfull et al. 2000) and in studies of horse domestication (e.g., Vila et al. 2001; Jansen et al. 2002). Polymorphisms in horse Y chromosome markers are not yet available at present, and efforts to detect variations in a wide variety of domestic horse breeds ended in failure (Wallner et al. 2003; Lindgren et al. 2004). In a recent study, 6 Y-specific microsatellite markers appeared as a haplotype block in domestic horse breeds (Wallner et al. 2004). China has a long history of horse breeding, and various local breeds have been shaped under different ecological circumstances. To date, more than 300 horse breeds have been identified worldwide, the single greatest number of which was indigenous to China (FAO 2007). These may be generally categorized into 3 major groups according to their origins, that is, North Pastoral Grassland, Northwest Plateau, and Southwest Mountain Area (Xie 1987).

In this study, Y chromosome microsatellites were used to analyze the patrilineal genetic variations of Chinese domestic horse breeds and their phylogeographic polymorphisms, and the Y-DNA haplotype diversities were investigated and confirmed by 2 sequencing methods. Polymorphisms in the horse breeds were highly geographically oriented. This work would promote the ancestry study of Chinese horse breeds in a new perspective.

## Materials and Methods

### Sample Collection and DNA Extraction

Blood samples from 531 animals were collected from 28 domestic horse breeds in 12 different provinces of China (Table 1) and from 2 exotic ones. In addition, 12 male Przewalski's horses (*Equus przewalskii*) from Xinjiang and

**Table 1** Name, code, sample size, and source region of 30 horse breeds

Distribution	Breed name	Code	N	Allele B of YA (F)	Source region
Middle and southeast of China	Jinjiang horse	JJ	11	2 (18.2%)	Jinjiang County, Fujian
	Lichuan horse	LCH	15	0	Lichuan City, Hubei
South of China	Debao pony	DBP	15	5 (33.3%)	Debao County, Guangxi
	Baise horse	BS	25	3 (12%)	Baise County, Guangxi
	Guizhou horse	GZ	15	5 (33.3%)	Anshun City, Guizhou
	Luoping horse	LP	18	2 (11.1%)	Luoping County, Yunnan
	Wenshan horse	WS	16	2 (12.5%)	Wenshan County, Yunnan
	Dali horse	DL	22	3 (13.6)	Jianchuan County, Yunnan
	Yunnan pony	YNP	12	2 (16.7%)	Gongshan County, Yunnan
	Jianchang horse	JC	19	2 (10.5%)	Butuo County, Sichuan
West of China	Tibet Valley horse	TRV	25	2 (8%)	Jiangzi County, Tibet
	Tibet Grassland horse	TGL	27	3 (11.1%)	Langkazi County, Tibet
	Yushu horse	YSH	15	0	Yushu City, Qinghai
Northwest of China	Hequ horse	HQ	10	0	Henan County, Qinghai
	Cadamu horse	CDM	20	0	Dulan County, Qinghai
	Datong horse	DT	20	0	Qilian County, Qinghai
	Yili horse	YL	21	0	Yili County, Xinjiang
	Yanqi horse	YQ	20	0	Yanqi County, Xinjiang
	Balikun horse	BLK	20	0	Yiwu County, Xinjiang
	Kazakh horse	KZK	25	0	Xinyuan County, Xinjiang
	Jilin horse	JL	15	0	Baicheng City, Jilin
Northeast of China	Elunchuns horse	ELC	13	0	Heihe City, Heilongjiang
	Heihe horse	HH	15	0	Sunwu County, Heilongjiang
	Sanhe horse	SH	14	0	Hulunbeie City, Inner Mongolia
	Sengseng horse	SS	10	0	Xilinguole City, Inner Mongolia
	Wuzhumuqin horse	WZH	16	0	Xilinguole City, Inner Mongolia
	Xinihe horse	XNH	15	0	Hulunbeie City, Inner Mongolia
	Mongolia horse A	MG	25	0	Hulunbeie City, Inner Mongolia
	Mongolia horse B	WMG	20	0	Bayandelger, Mongolia
Abroad breeds	Thoroughbred horse	THB	15	0	Beijing, China (introduced from UK)

N, number of samples; YA, Eca.YA16; F, frequency.

30 male donkeys (*Equus asinus*) from Yunnan in China were incorporated. Five female horses were used as negative control for the identification of 6 Y chromosome markers. Genomic DNA was extracted from all blood samples using a standard phenol–chloroform extraction method (Sambrook et al. 1989).

### Microsatellite Loci and Genotyping

Six Y chromosome microsatellite loci were chosen to analyze the genetic variations of the domestic horse breeds, Przewalski's horse and donkey (Wallner et al. 2004). Forward primers were 5'-labeled with fluorescent dyes (FAM or HEX). The relevant information corresponding to these loci is as shown in Supplementary Material Table S1. Genotype for each marker was determined using an ABI 3130 DNA Sequencer (Applied Biosystems) with GeneScan™ LIZI 500 internal size standards (Applied Biosystems).

Polymerase chain reaction (PCR) amplifications were performed in 12- $\mu$ l reaction volumes each containing 50 ng of genomic DNA, 2.5 mM MgCl<sub>2</sub>, 250  $\mu$ M of dNTP, 0.025  $\mu$ M of both forward and reverse primers, 1.25 units of *Taq* polymerase, and 1 $\times$  magnesium-free PCR buffer (Takara, Kyoto, Japan). Amplifications were carried out

using the GeneAmp PCR 9700 thermocycler (Applied Biosystems), with the following PCR cycle: 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, annealing at 54–60 °C (Supplementary Material Table S1) for 30 s, 72 °C for 30 s, and a final step at 72 °C for 10 min. PCR products were diluted 2- to 4-fold, 0.75  $\mu$ l of which was then mixed with an internal standard (GeneScan™ LIZI 500, Applied Biosystems) according to the manufacturer's instructions. Genotyping was carried out on an ABI 3130xl automated capillary sequencer. Sequence analysis was performed using GeneMapper V3.7 software (Applied Biosystems). The third-order least square method was used for allele size determination (Mburu et al. 2003). Genotyping was repeated once when any individual samples failed to be amplified. Samples with mutant allele were subject to PCR amplifications and electrophoresis for a second time to verify the type.

### Sequencing Mutant Alleles

Direct sequencing and cloning sequencing were applied combinatorially to acquire credible results, by means of which 2 samples of each haplotype were sequenced in each breed which contains mutant allele for direct sequencing. PCR reaction was carried out in a 60- $\mu$ l reaction volume. The settings



**Figure 1.** The geographic distribution and sample sizes of Allele A and Allele B horse breeds. 1) Jinjiang horse (JJ); 2) Lichuan horse (LCH); 3) Debao pony (DBP); 4) Baise horse (BS); 5) Guizhou horse (GZ); 6) Luoping horse (LP); 7) Wenshan horse (WS); 8) Dali horse (DL); 9) Yunnan pony (YNP); 10) Jianchang horse (JC); 11) Tibet Valley horse (TRV); 12) Tibet Grassland horse (TGL); 13) Yushu horse (YSH); 14) Hequ horse (HQ); 15) Chaidamu horse (CDM); 16) Datong horse (DT); 17) Yili horse (YL); 18) Yanqi horse (YQ); 19) Balikun horse (BLK); 20) Kazakh horse (KZK); 21) Jilin horse (JL); 22) Elenchuns horse (ELC); 23) Heihe horse (HH); 24) Sanhe horse (SH); 25) Sengseng horse (SS); 26) Wuzhumuqin horse (WZH); 27) Xinihe horse (XNH); 28) Mongolia horse A (MG); 29) Mongolia horse B (WMG); 30) Thoroughbred horse (THB). *n*, number of samples; (TG)<sub>3</sub>TA (TG)<sub>18</sub>, Allele A; (TG)<sub>3</sub>TA (TG)<sub>16</sub>, Allele B.

were an initial denaturation at 94 °C for 5 min, followed by 20 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s and a final extension of 10 min at 72 °C. Amplified DNA segments were subject to electrophoresis on a 1% agarose gel.

The PCR products were gel purified with an OMEGA gel extraction kit (OMEGA) and cloned into a pEasy-T1 vector (TransGen, Beijing, China), which was subsequently used to transform *Escherichia coli*. The double-strand DNA inserted into the vector was completely sequenced. At least 3 clones per sample were selected for sequencing. Meanwhile, direct sequencing was performed in order to verify the reliability of the sequencing results for the same segments with an ABI 3730xl automatic sequencer (Applied Biosystems).

## Results

Genetic variations of the 6 equine Y-specific microsatellites were assessed in the 531 male domestic horses, 12 male Przewalski horses, and 30 domestic donkeys. PCR products of male samples exhibited a single band, whereas none in the female samples. The 5 Y-specific microsatellites (Eca.YH12, Eca.YM2, Eca.YP9, Eca.YE1, and Eca.YJ10) displayed no variations in the domestic horse breeds. Two haplotypes of Eca.YA16, Haplotype A (Allele A: 156 bp)

and Haplotype B (Allele B: 152 bp), however, were discovered. Each haplotype was exclusively detected in a specific individual. Allele A of Eca.YA16 was a common allele appearing in most domestic horse breeds (Wallner et al. 2004). The Przewalski's horse (*n* = 12) alleles differed from those of the domestic horse breeds at 1 locus (Eca.YA16: 158 bp; Supplementary Material Table S1). All the 6 loci showed a single haplotype in the domestic donkey, 4 (Eca.YH12, Eca.YM2, Eca.YP9, and Eca.YE1) of which showed different alleles in donkey from those in horse. Only Eca.YJ10 locus displayed exactly the same allele (213 bp) in the horses and donkeys. The allele of donkey in Eca.YA16 was the same as Allele A (156 bp) of horse.

There were 2 haplotypes in Eca.YA16 among the domestic horse breeds. This is the first time that polymorphism was discovered in horse Y-DNA, and the reliability of the results was further verified with 2 methods. There existed Allele A (Eca.YA16: 156 bp) and Allele B (Eca.YA16: 152 bp) in the domestic horse breeds. The sequencing results also proved that there were 4 base differences between the 2 haplotypes. As shown in results of sequencing, the segment (TG)<sub>3</sub>TA(TG)<sub>18</sub> corresponded to PCR product of Allele A (Eca.YA16: 156 bp) and the repeat (TG)<sub>3</sub>TA(TG)<sub>16</sub> represented the PCR products of Allele B (Eca.YA16: 152 bp). Allele A was 2 copies of TG longer than Allele B.

The numbers and proportions of samples with Allele A and Allele B from all breeds were calculated. Allele A in Eca.YA16 was the common allele of 30 horse breeds, whereas Allele B in only 11 horse breeds. Thirty-one samples from 11 horse breeds, 5.85% out of the 530, were found with Allele B in Eca.YA16 (Table 1). A clear distribution pattern of the 2 haplogroups was observed (Figure 1), implying that the horse breeds with Allele B were highly geographically oriented. And differences among the breeds in the south of the Yangtze River region in China account for the major part of the polymorphism.

## Discussion

Markers in nonrecombinant regions are linked throughout the entire length of the Y chromosome. A set of linked microsatellite markers records the evolutionary history of a particular Y chromosome on which it is located and is an important tool for investigating populations or evolutionary processes. Y-specific polymorphic microsatellite markers have been used to develop a number of haplotypes that reflect ancient as well as recent evolutionary events in the history of human evolution (Whitfield et al. 1995; Shen et al. 2000; Mukherjee et al. 2001; Stumpf and Goldstein 2001; Saha et al. 2003; Cordaux et al. 2004). The presence of a single Y chromosome haplotype in the domestic horse breeds suggests a significantly lower contribution to the gene pool of the domestic horse by stallions compared with the mares.

Though the 6 Y-specific microsatellites displayed limited genetic variation in the domestic horse breeds, this was the first time polymorphism was found in nonrecombinant horse Y-DNA. Moreover, Chinese domestic horse breeds carrying 2 alleles were only in the southern area of the Yangtze River, mainly in the southwest of China. Horse breeds in these regions have distinct appearances from those in the North of China. The adult individuals of many horse breeds in Yunnan, Guizhou, Sichuan, and Guangxi provinces, for example, Baise horse, Debao Pony, Jianchang horse, etc., have a height lower than 1 m, which have been used in tourism.

Horse breeds in the southwest of China showed highly polymorphic blood protein markers and genomic DNA markers (Hou et al. 1993; Wang et al. 2001). In the report of Wang et al. (2001), polymorphisms were detected at 4 short tandem repeat through polyacrylamide gel electrophoresis of serum samples from 16 Chinese miniature horses. In addition, the molecular evidence of Tibetan horses indicated that Tibetan horse population (located in the southwest of China) might have multiple origins (Xu et al. 2007). Two Y-DNA haplogroups were discovered in the domestic horse breeds in China, and this can provide useful insights into the evolutionary mechanisms of polymorphisms for this chromosome and is helpful in unveiling the intricate genetic genealogy of horse breeds.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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