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RESEARCH ARTICLE

Y-chromosome polymorphisms of the domestic Bactrian camel in China

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Abstract. Single-nucleotide polymorphisms (SNPs), microsatellites and copy number variation (CNV) were studied on the Y chromosome to understand the paternal origin and phylogenetic relationships for resource protection, rational development and utilization of the domestic Bactrian camel in China. Our sample set consisted of 94 Chinese domestic Bactrian camels from four regions (Inner Mongolia, Gansu, Qinghai and Xinjiang), we screened 29 Y-chromosome-specific loci for SNPs, analysed 40 bovine-derived microsatellite loci and measured CNVs of *HSFY* and *SRY* through Sanger sequencing, automated fluorescence-based microsatellite analysis and quantitative real-time PCR, respectively. A multicopy gene, *SRY*, was first found, and sequence variation was only detected in *SRY* in a screen of 29 loci in 13 DNA pools of individual camels. In addition, a TG repeat in the *USP9Y* gene was identified as the first polymorphic microsatellite in the camel Y chromosome, whereas microsatellite based on bovine sequences were not detected. The frequency of each allele varied among different populations. For the Nanjiang, Hexi and Alashan populations, a 243-bp allele was found. For the Sunite population, 241-bp, 243-bp and 247-bp alleles were detected, and the frequencies of these alleles were 22.2%, 44.5% and 33.3%, respectively; 241-bp and 243-bp alleles were found in other populations. Finally, CNVs in two Y-chromosomal genes were detected; CNV for *HSFY* and *SRY* ranged from 1 to 3 and from 1 to 9, respectively.

Keywords. Bactrian camel; Y chromosome; single-nucleotide polymorphisms; microsatellite; copy number variation.

Introduction

As a precious species resource and an indispensable animal in Chinese agricultural production, the domestic Bactrian camel plays an important role in economic trade, cultural construction and national defence. Recently, the rapid development of modern transportation and agriculture, the lagging development of camel-derived products and environmental degradation have led the camel industry to grow slowly, and the number of camels has decreased drastically, resulting in the depletion of germplasm resources. Therefore, the genetic diversity of Bactrian camels need to be studied to clarify its phylogenetic relationships and lay the foundation for conserving its biodiversity. To date there have been many reports on the maternal origin of domestic Bactrian camels in China. Numerous studies have shown that there is abundant sequence diversity and no significant differences among populations of domestic Bactrian camels in China. Domestic Bactrian camels in China were clustered into one group, which indicated a single-maternal origin (Cheng *et al*. 2009; Ji *et al*. 2009; Zhang *et al*. 2015a). However, mtDNA polymorphisms only indicate maternal origin and to date there has been no research on the paternal contributions on domestic Bactrian camels using Y-chromosome molecular markers.

Because of the strict paternal inheritance, nonrecombination with the X chromosome, low mutation rate, and easy formation of specific haploids, molecular markers specific to male-specific regions on the Y chromosome can provide effective information for exploring phylogenetic relationships among populations. Recently, Y-SNP (single-nucleotide polymorphisms on the Y chromosome) and Y-STR (short-tandem repeat on the Y chromosome) variants have been widely used to analyse the origins of

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Table 1. Sample information for domestic Bactrian camels in China.

Population	Code	Sampling site	Sample size
Dongjiang camel	DJ	Mulei county, Xinjiang	17
Nanjiang camel	NJ	Wensu county, Xinjiang	12
Beijiang camel	BJ	Qinghe county, Xinjiang	15
Hexi camel	HX	Yongchang county, Gansu	5
Qinghai camel	QH	Mohe, Qinghai	19
Alashan camel	ALS	Alashan Left Banner, Inner Mongolia	8
Sunite camel	SNT	Sunite Right Banner, Inner Mongolia	18
Total			94

many animals and humans (Natanaelsson *et al*. 2006; Cliffe *et al*. 2010; Zhang *et al*. 2014; Han *et al*. 2015). For example, two paternal lines in Przewalski's horse were detected (Wallner *et al*. 2004; Kreutzmann *et al*. 2014), and the phylogeny reflected a sister taxa between Przewalski's horse and domestic horses (Wallner *et al*. 2003). Chinese cattle have only Y2 and Y3 paternal lineages (the Y1 haplogroup is mainly distributed in northwest Europe) (Li *et al*. 2013), consistent with mtDNA evidence, historical record and geographical distribution (Lei *et al*. 2006). The analysis of Y chromosome sequence variation showed that humans originated in Africa (Underhill *et al*. 2000), which coincided with historical record and archaeological data. Therefore, Y-chromosome markers are necessary for unveiling paternal origin.

Copy number variation (CNV) can also reflect individual differences in genetic background (Zhang *et al*. 2015b). Currently, CNV on the Y chromosome for domestic Bactrian camels in China remains unknown. Therefore, detection of molecular markers on the Y chromosome becomes feasible and is essential for laying a foundation for determining paternal origin, resource protection and utilization of the domestic Bactrian camel in China.

Materials and methods

Sample collection and DNA extraction

In this study, blood samples of 94 male domestic Bactrian camels from Inner Mongolia, Gansu, Qinghai, and Xinjiang were used (table 1; figure 1). Additionally, to verify the male specificity of primers, two female camels were also collected as negative controls. DNA samples were extracted using a standard phenol–chloroform method (Sambrook and Russell 2002) and were diluted to 20 ng/μL.

SNP detection

Twenty-nine pairs of primers were designed to amplify Y-chromosome-specific fragments of domestic Bactrian camel DNA using Primer Premier software (ver. 5.0). The primers were based on the Y chromosome sequences of wild Bactrian camels (table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/) and were diluted to 10 μM.

A total of 13 DNA pools, each comprising 6 to 10 individuals (5 μL of DNA per individual) were used as templates for polymerase chain reaction (PCR) amplification to screen SNPs in a 14.2 kb Y-chromosome sequence divided into 29 fragments. PCR reactions were performed at a 10 μL volume containing 0.5 μL genomic DNA, 0.2 μL of each primer, and 5 μL Ex Taq Master Mix (CWBio, Beijing, China). The PCR protocol included the following steps: denaturation at 95°C for 5 min, followed by 34 cycles of 94°C for 30 s, annealing for 40 s, and 72°C for 40 s, with a final extension at 72°C for 10 min. The following steps were conducted for the Touchdown PCR protocol: denaturation at 94°C for 5 min, followed by 10 cycles of 94°C for 30 s, 65°C to 55°C for 40 s, and 72°C for 50 s, followed by 25 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 50 s, and a final extension at 72°C for 10 min. The PCR products from the DNA pools were amplified by 29 pairs of primers that were directly sequenced by Sanger sequencing (Shanghai Sangon Biotech, Shanghai, China), and with the default values of parameters set up, all sequences were aligned by DNASTAR 5.0 (DNASTAR, Madison, USA) to determine polymorphic sites.

To verify the presence of *SRY* gene SNPs, PCR products from every sample in the DNA pools amplified by *SRY* primers were directly sequenced and these sequences were aligned. Because the *SRY* sequence of some individuals had the same base substitution and indel sites as the DNA pools, one sample from each population was selected for amplification to test the accuracy of these results. The PCR products were cloned into pGEM-T vectors using the pGEM-T Easy Vector system I (Promega, Madison, USA). The vectors were transferred into *Escherichia coli* DH5α competent cells (TaKaRa Bio, China) according to the manufacturer's instructions. For each sample, 10 colonies were selected for sequencing. All sequences were aligned with DNASTAR 5.0.

Figure 1. A geographical map of the sampling sites.

Microsatellite detection

Based on the Y chromosome sequence of wild Bactrian camels, a pair of primers was designed to amplify a Y chromosome-specific microsatellite: USP9Y-STR forward primer: 5′-TTGGCTGTGGGGTTTTCTT-3′, USP9Y-STR reverse primer: 5′-TTTCCCCGTGAAGAATGCT-3′. In addition, 39 pairs of primers for bovine Y chromosome microsatellites (Liu *et al*. 2002, 2004) were used and are shown in table 2 in electronic supplementary material. All of the primers were verified to be male specific by conducting PCR containing two female camel DNA samples along with two male samples. PCR reactions were performed in 8 μL volumes containing 0.96 μL genomic DNA, 0.16 μL of each 10 μM primer, 4 μL 2× Type-it Multiplex PCR master mix and 2.72 μL RNase-free water (Qiagen, Valencia, USA). PCR was conducted using the following conditions: denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55–66°C for 90 s, and 72°C for 30 s, with a final extension at 60°C for 30 min.

The male-specific product was further cloned and sequenced to confirm the presence of a 19-TG-repeat microsatellite. Subsequently, the forward primer, which was male-specific, was fluorescently labelled with FAM (6-carboxy-fluorescein) at the 5′ end. Using the same PCR programme amplification was performed, and the products from 94 male samples along with the female DNA controls were separated by capillary electrophoresis (Shanghai Sangon Biotech Company). Allele size and frequency were determined.

Table 2. The allele frequency of USP9Y-STR and median CN of *HSFY* and *SRY* in seven groups.

Group	Allele frequency (bp)			Median CN	
	241	243	247	*HSFY*	*SRY*
DJ	0.353	0.647	0.000	1 (1-2)	4 (1-5)
BJ	0.200	0.800	0.000	1 (1-2)	5 (2-6)
NJ	0.000	1.000	0.000	1 (1-2)	4 (3-8)
HX	0.000	1.000	0.000	2 (1-2)	5 (2-8)
QH	0.056	0.944	0.000	1 (1-3)	4 (1-5)
ALS	0.000	1.000	0.000	1 (1-2)	4 (3-6)
SNT	0.222	0.445	0.333	2 (1-2)	4 (1-9)
Total	0.149	0.787	0.064	1 (1-3)	4 (1-9)

CN detection

Because there was no previous report on single-copy genes on the Y chromosome, the single-copy gene *CYP2A* on the

autosome was used as a reference (Jirimutu *et al.* 2012). The primer sequences of *HSFY*, *SRY* and *CYP2A* are shown in table 3 in electronic supplementary material. The primers were also verified to be male specific by including DNA from two female camels along with two male camel DNA samples. PCR parameters were identical to those used for SNP detection.

Quantitative real-time PCR (qPCR) was used to measure the CN of the *HSFY* and *SRY* genes in the samples using the Bio-Rad CFX-96 real-time PCR system and the SYBR Premix Ex Taq kit (TaKaRa, China). Standard curves were generated from high-quality genomic DNA diluted to 60, 40, 20, 10 and 5 ng/μL for the *HSFY*, *SRY* and *CYP2A* primers. DNA of the test camel samples was concentrated to 5 ng/μL. Samples for standard curves and test samples were prepared in triplicate for each concentration (including the calibrator for each plate). Each PCR reaction (10 μL) consisted of 5 μL SYBR Premix Ex Taq II (Tli RNaseH Plus) (2×), 0.8 μL DNA and 0.4 μL of each 10 μM primer. The qPCR protocol included predenaturation at 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 5 s, annealing for 30 s, and one cycle of denaturation at 95°C for 10 s and 65°C for 5 s. A melting curve was then generated by taking fluorescence measurements every 0.5°C from 65°C until 95°C. The CNs for *HSFY* and *SRY* were determined for test samples using the following equations introduced in Hamilton *et al.* (2009):

$$E = 10^{-1/\text{slope}} \tag{1}$$

$$\text{CN calibrator} = (E_{\text{reference}})^{\text{CTreference}} / (E_{\text{target}})^{\text{CTtarget}} \tag{2}$$

$$\text{Ratio} = (E_{\text{target}})^{\triangle\text{CTtarget(calibrator}-\text{sample)}} / (E_{\text{reference}})^{\triangle\text{CTreference(calibrator}-\text{sample)}} \tag{3}$$

$$\text{CN sample} = (\text{CN of the calibrator}) \times (\text{ratio}). \tag{4}$$

Normal distribution of the *HSFY* and *SRY* CN data was assessed with the Kolmogorov–Smirnov test using SPSS 17.0 software (SPSS, Chicago, USA).

Results

SNP detection

The male specificity for PCR amplification of 29 pairs of primers was confirmed. For the PCR products of the vast majority of primers, all of the samples carried the same Y-chromosome haplotype, whereas a base substitution and indel sites were found in the *SRY* gene (figure 2a). Subsequently, PCR products from every single sample in the DNA pool amplified by the *SRY* primers were directly sequenced, which showed that the *SRY* sequence of some individuals had the same base substitution and indel sites as the DNA pool. Thus, the *SRY* gene was suspected to be multicopy. To test the accuracy of these results, one sample from each population was selected for amplification, and the PCR products were cloned and sequenced. Mutation sites among different colonies from the same sample were observed, which demonstrated that there was a multicopy *SRY* gene in domestic Bactrian camels (figure 2b).

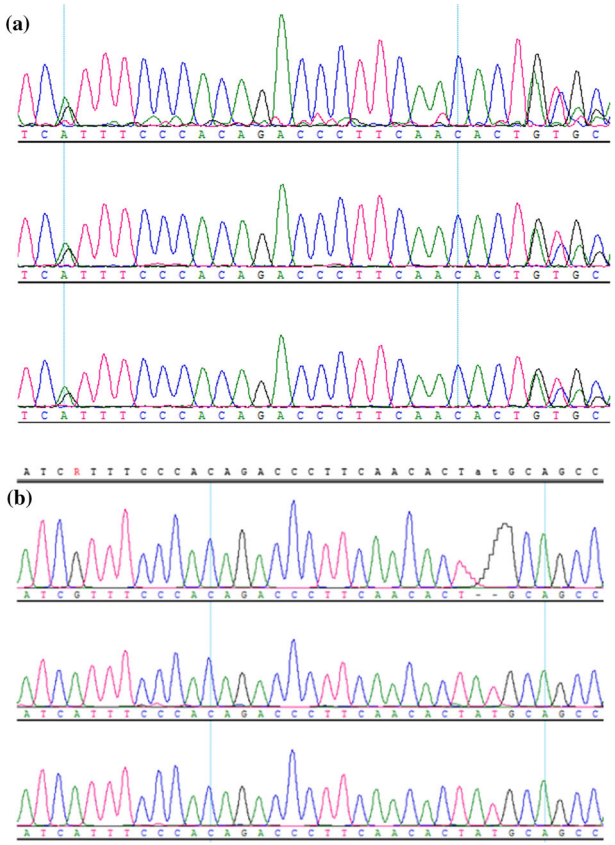

Figure 2. (a) The *SRY* sequences of DNA pools and (b) different colonies of the *SRY* gene from the same sample.

Microsatellite detection

Of the 40 STRs that were screened, only USP9Y-STR was male-specific. The male-specific product was further cloned and sequenced, and a 19 dinucleotide TG repeat was found, indicating that USP9Y-STR was a microsatellite on the Y chromosome (figure 3). With female DNA used as a negative control, the capillary electrophoresis results showed that USP9Y-STR was a polymorphic microsatellite (figure 4).

A total of three alleles were detected for USP9Y-STR, which were 241 bp, 243 bp and 247 bp. Overall, the 243-bp allele was found in 78.7% of the samples from the seven populations, higher than the other alleles, indicating that it was the major allele. In addition, the frequencies of different alleles in the seven populations varied. For

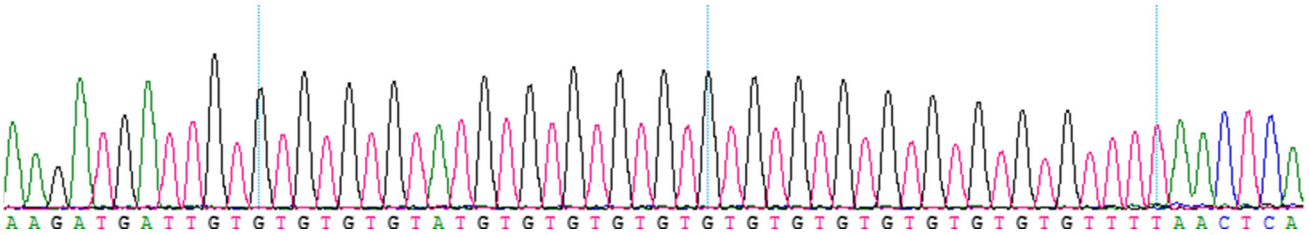

Figure 3. Sequence chromas of USP9Y-STR.

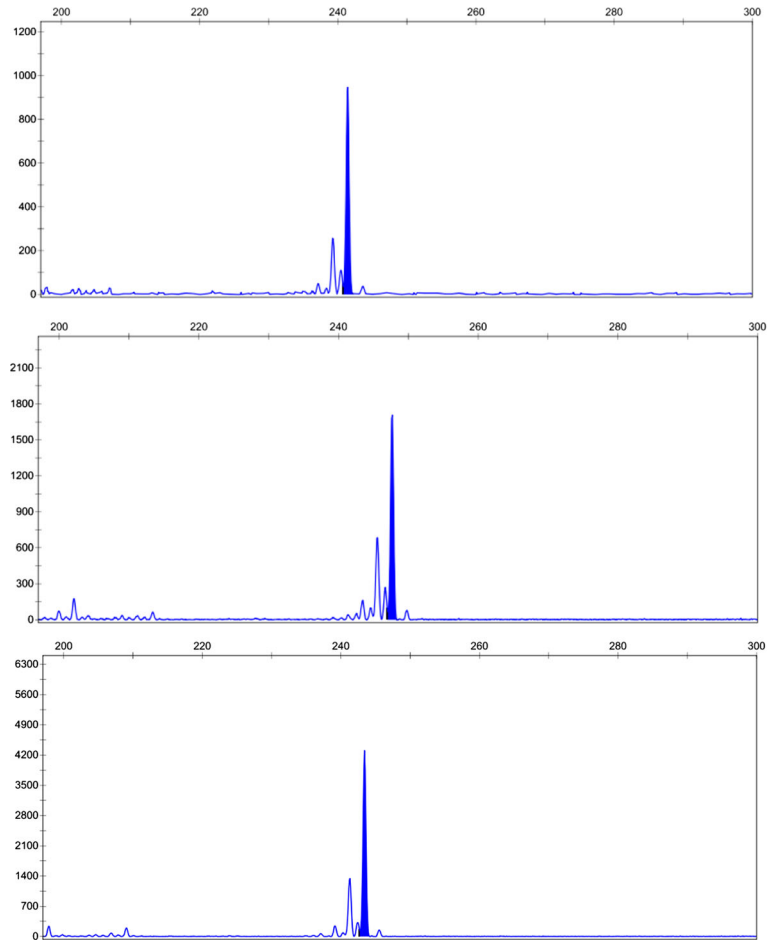

Figure 4. Gene scanning of the PCR product for USP9Y-STR.

Nanjiang, Hexi and Alashan populations, only the 243-bp allele was found. For the Sunite population, the 241-bp, 243-bp and 247-bp alleles were detected, and the frequencies were 22.2%, 44.5% and 33.3%, respectively. We found 241-bp and 243-bp alleles for other populations as well (table 2).

For the Xinjiang population, the 243-bp allele was dominant and the frequency was 79.5% and the 241-bp allele accounted for 20.5%. In Inner Mongolia, the 241-bp, 243-bp and 247-bp alleles accounted for 15.4%, 61.5% and 23.1%, respectively.

CNV of HSFY and SRY

The CN of *HSFY* in the calibrator was estimated to be 1. Subsequently, the CN of the remaining samples was calculated relative to the calibrator. The median CN of *HSFY* was 1, ranging from 1 to 3 among all the domestic Bactrian camels. The CN of *SRY* in the calibrator was estimated to be 2. The median CN of *SRY* was 4, ranging from 1 to 9 among all domestic Bactrian camels (table 2).

We found that the CN data of *HSFY* and *SRY* did not follow a normal distribution in 94 samples as well as in

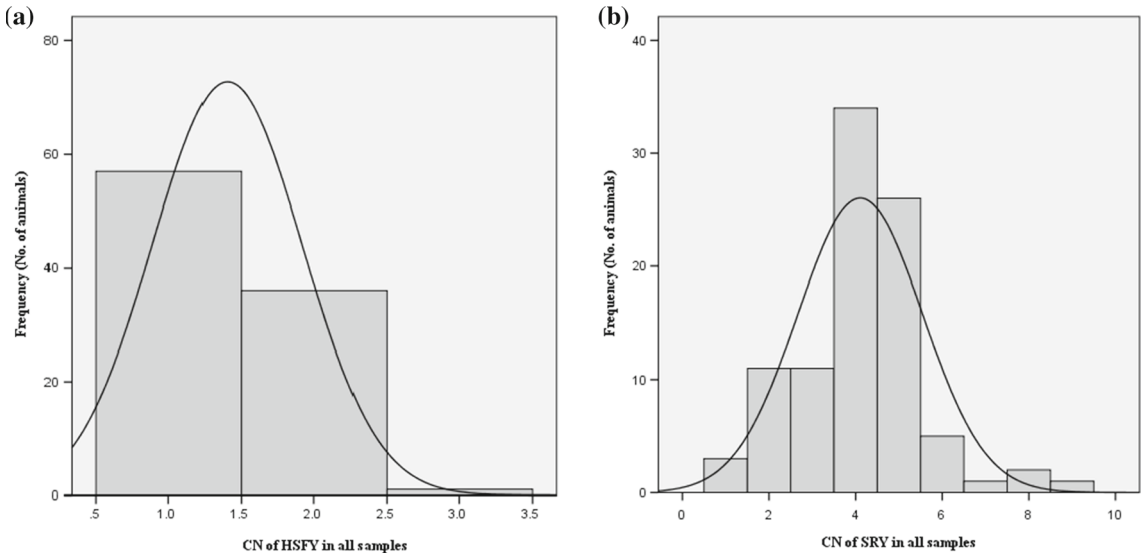

Figure 5. Distribution of (a) *HSFY* and (b) *SRY* CNs in all samples.

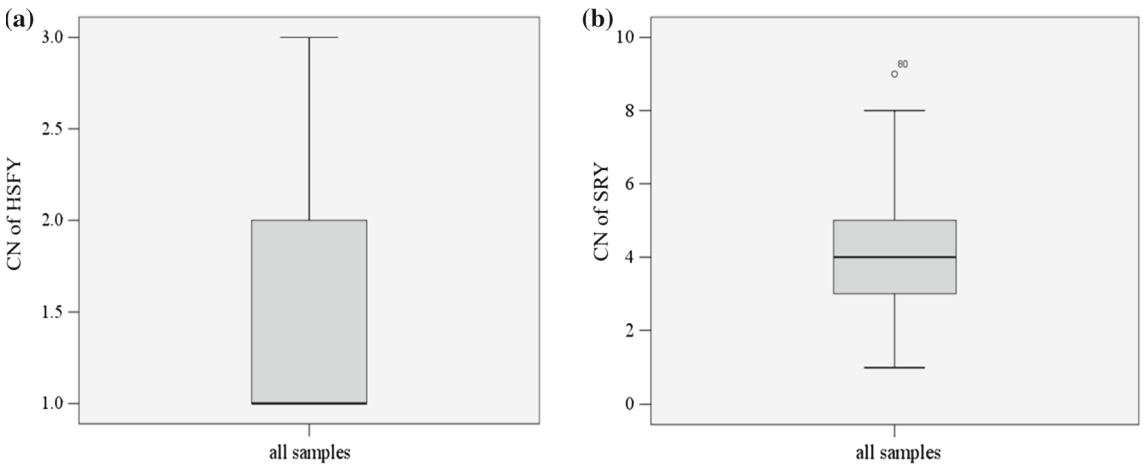

Figure 6. Box plot results of *HSFY* and *SRY* CNs in the total population.

each population based on the Kolmogorov–Smirnov normality test (figures 5; figure 1 in electronic supplementary material).

Discussion

The genetic diversity and the evolutionary origin of domestic Bactrian camels in China have been a source of fascination for decades. To date, most experts have focussed on studying polymorphisms of autosomes and mtDNA to uncover the phylogenetic relationship among populations of domestic Bactrian camels in China, while there have been no reports on the paternal contributions of domestic Bactrian camels using Y-chromosome molecular markers. Therefore, we analysed the Y-SNP, Y-STR and Y-CNV of domestic Bactrian camels. A total of 29 pairs of Y-chromosome-specific primers were screened for SNPs, but the PCR products of a vast majority of primers had the same Y-chromosome haplotype. This outcome can probably be explained by the fact that some SNPs could have been missed and not identified during screening of the DNA pools leading to underestimation of SNPs (Vollmer and Rosel 2012).

A TG repeat in the *USP9Y* gene was identified as the first polymorphic microsatellite in the camel Y chromosome. Currently, cross-species amplification of known microsatellite primers has been the most popular approach for studying Y-chromosome-specific microsatellite markers of different species. For example, some Y-chromosomal microsatellite markers identified from the human genome have been applied to studies of gorillas, hamadryas baboons, and other species (Hammond *et al*. 2006; Douadi *et al*. 2007). Similar to those studies, polymorphic microsatellites on the Y chromosome in the domestic dog have been successfully amplified in wolves (Musiani *et al*.

2007). In the present study, 39 bovine Y-chromosome microsatellites were tested for amplification in domestic Bactrian camels and no male-specific products were found, which indicated that these primers were not suitable for detecting microsatellites in Chinese domestic Bactrian camels. This amplification profile is most likely related to the evolutionary distance between Bactrian camel and cattle, which diverged approximately 55–60 million years ago (Ji *et al*. 2013) and led to the accumulation of a large number of mutations in the primer-binding sites as well as a decrease in the overall PCR efficiency. This resulted in the frequently observed problem of nonspecific binding. In addition, this problem can be further aggravated by the characteristics of the Y-chromosome sequence such as its highly repetitive structure.

qPCR has been widely used for the detection of gene CN (Giachini *et al*. 2009; Mukherjee *et al*. 2013; Yue *et al*. 2014), and the results from this study were consistent with the predictions. Therefore, this approach was feasible for this experiment. In the present study, we first investigated the CNV of *HSFY* and *SRY* of domestic Bactrian camels in China using qPCR. *HSFY* CN ranged from 1 to 3, indicating that the *HSFY* gene was a multicopy gene in domestic bactrian camels in China. To date, numerous studies have shown that the feline Y chromosome possesses eight copies of *HSFY* (Wilkerson *et al*. 2008), and humans have two functional copies and four similar copies (82% homology to *HSFY*) (Helen *et al*. 2003; Tessari *et al*. 2004). The *HSFY* CN was greatly expanded in cattle and pig lineages, and cattle had between 21–308 copies. The CN within and between breeds significantly varied (Yue *et al*. 2014), while the *HSFY* CN was 100 for pig lineages (Skinner *et al*. 2015). The *HSFY* CN in domestic Bactrian camels in China is low compared to that of other mammals. The *SRY* CN was found to range from 1 to 9. Compared to the results reported in other mammals, the *SRY* CN also varied. For example, the *SRY* gene had one copy in humans, horses, cattle and pigs (Skaletsky *et al*. 2003; Paria *et al*. 2011; Chang *et al*. 2013), whereas it was multicopied in the domestic cat, with up to four copies (Pearks *et al*. 2008). In *Rattus norvegicus*, the Y-chromosome accumulated 11 copies of the *SRY* loci, and there were at least six copies of the *SRY* loci that were functional (Turner *et al*. 2007; Prokop *et al*. 2013). The difference in CN further demonstrated the heterogeneity of the Y chromosome among species. Additionally, the deviation from normal distribution was probably related to the small number of gene copies that were involved and the differentiation of populations.

These results show that allele 247 is unique to the Sunite population, and the *SRY* locus has a very high CN in this population. This outcome may have resulted from geographic distribution because the Yinshan Mountains create a natural barrier that hinders genetic exchange between the Sunite population and other populations. Further, camel breeding projects for improving wool production were carried out in the Sunite region. For example, excellent male camels were introduced and mated with the breeding population to avoid inbreeding, which resulted in improvements in the wool production and economic benefits (Zhang *et al*. 1991). Therefore, geographic distribution and directional selection may give rise to population differentiation. In addition, this differentiation can be attributed to a distinct paternal ancestry expected for this population, but more studies on the Y chromosome of the Bactrian camel in China are needed in the future.

Due to the low number of Y-chromosome-specific genes and microsatellites included in this study and the lack of related information concerning Y chromosome polymorphisms of domestic Bactrian camels, this study is insufficient to comprehensively understand the paternal origins of Chinese domestic Bactrian camels. Therefore, to comprehensively reveal phylogenetic relationships among populations, DNA samples should be collected from foreign camels and the population sizes need to be expanded. Further, we need more powerful methods, such as high-throughput sequencing to discover additional polymorphic markers on the Y chromosome.

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