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Asian Journal of Animal and Veterinary Advances



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## **Genetic Diversity of Tibetan Horse and its Relationships with Mongolian Horse and Ningqiang Pony Assessed by Microsatellite Polymorphism**

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### **ABSTRACT**

Tibetan Horse lives in Qinghai-Tibet Plateau of South-West China, the controversy on whether Tibetan Horse should be classified into Southwest Horse Type or not has existed in China for a long time as there are four other type horses in the country. In the study, the genetic diversity and structure of Tibetan Horse were investigated using 14 microsatellite markers and 153 animals belonging to three Tibetan Horse subpopulations in Xigaze, Qamdo and Nagqu, respectively and Mongolian Horse and Ningqiang Pony which served as reference groups to Tibetan Horse. The results showed that the mean Number of Allele (MNA) across the 14 loci in Tibetan Horse was 13.1 and the average observed heterozygosity (0.710) and PIC (0.752) were slightly higher than those (0.692 and 0.773, respectively) of Mongolian Horse. Although, there was abundant genetic variation, genetic differentiation between horse populations was low, with only 2.1% of the total genetic variance among populations. Qamdo, Xigaze and Mongolian Horse had close relationship and Nagqu is distinctly separated from the other two Tibetan Horse subpopulations and referenced breeds in the clusters. The results indicated the genetic relationship of Tibetan Horse were closer to Mongolian Horse rather than to Ningqiang Pony, a typical breed in Southwest Horse Type, which suggests that Tibetan Horse was a unique horse breed which could not be classified into Southwest Horse Type.

**Key words:** Genetic diversity, relationship, Tibetan horse, Mongolian horse, Ningqiang Pony, microsatellite marker

### **INTRODUCTION**

There are five indigenous horse types in China, Tibetan Horse, Mongolian Horse, Southwest Horse, Hequ Horse and Kazakh Horse which were classified based on body conformation, distribution and history (Xie, 1986). Tibetan Horse, distributing around in four Southwest Provinces of China, Tibet, Qinghai, Sichuan and Yunnan, 60% of total live in Tibet. Tibetan Horse is classified into three different subpopulations as follows, Hill Type, accounting for 32% of total Tibetan Horse Population and in Qamdo prefecture; Altiplano Type, occupying 44% of the total and in Nagqu prefecture; Valley Type, making up 18.8% of the total and in Xigaze city (Xie, 1986).

Tibetan Horse is well adapted to high altitude in Qinghai-Tibet Plateau and extremely hardy and disease-resistant. Although, it lives in Southwest China, but some researchers classify it into

a unique horse breed, while others believe it should be a breed in Southwest Horse Type together with other horse populations existing in Southwest region (Chang, 1995; Xie, 1986). Finally, the classification of Tibetan Horse remains controversial.

Mongolian Horse mainly distributes in Inner Mongolia region of Northern China, which had played an important role in world's history. The great expansions of the Mongolian Empire under Attila (5th century) and Genghis Khan (13th century) resulted in the migrations of both its people and horses into its new territory, including Tibet (Sheng and Liu, 2006). Close relationship between the Mongolian native horse and Northern European horse breeds was observed by Bjornstad *et al.* (2002), thus there was a possible linkage between Mongolian Horse and Tibetan native horse.

The relationship among the three Chinese indigenous horse breeds, Tibetan, Mongolian and Southwest Horse, remains unclear up to now. In this study, Ningqiang Pony was referred to as Southwest Horse breeds. Though there are some reports on Mongolian Horse or Southwest Horse (Li *et al.*, 2005; Sun, 2008), the genetic research on Tibetan Horse is absent. The only report on it was the research conducted for the zoological characteristics and polymorphism of blood proteins so far (Wang, 1996). The research of other livestock (chicken in Yu *et al.*, 2006; sheep in Zhang *et al.*, 2008b; goat in Mahmoudi, 2010) demonstrated that microsatellite is a useful tool for studying the genetic relationship among closely related breeds. In the study, we studied the genetic divergence of Tibetan Horse in Tibet using 14 microsatellite loci recommended by FAO and ISAG, which was the first time to study the three Tibetan horse subpopulations together as far as we know. It may reveal the genetic relationship among Tibetan Horse, North Horse and Southwest Horse. The information generated in this study may further be utilized for studying differentiation and relationship among different Chinese indigenous horse breeds.

## MATERIALS AND METHODS

**Sampling collection:** This research project was fully sponsored by National Basic Research Program of China with grant number 2006CB102100. A total of 239 animals were sampled from Mongolian Horse in 2006, Ningqiang Pony in 2007 and the three Tibetan Horse populations in 2007. Blood or ear skin was randomly collected. The sampled populations and their geological information are shown in Table 1 and Fig. 1, respectively. Horse DNA was isolated from whole blood or ear tissues in Equine blood testing center of China Agricultural University (Beijing) according to a modified phenol-chloroform extraction method as Sambrook and Russell (2002) noted.

**Microsatellite markers and genotyping:** Fourteen microsatellite markers (AHT4, ASB17, ASB23, HTG7, HMS2, HTG6, HMS6, HTG10, LEX33, ASB2, VHL20, HMS3, HMS7, HTG4) dispersed over 10 different chromosomes were used in the present study. Each of the

Table 1: Sampling information of the studied horse breeds

Breed	Geographical location	No. of animals	Sample
Tibetan horse	Xigaze	61	Ear skin
	Nagqu	39	Ear skin
	Qamdo	53	Ear skin
Mongolian horse	Chifeng city in Inner Mongolia	50	Blood
Ningqiang Pony	Ningqiang town in Shanxi Province	36	Blood



Fig. 1: Geographical location of the five horse breeds

14 microsatellites was amplified alone in independent PCR reaction. Forward primers were end-labelled with fluorescent dyes (6-FAM, TET or HEX). Genotypes for each marker were determined at State Key Laboratory of Agrobiotechnology (Beijing) using ABI 377 DNA Sequencer (Applied Biosystems) with the internal size standard GeneScan™-TAMARA350 (Applied Biosystems).

**Statistic analysis:** Allele frequency, the number of alleles per locus, observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) for each breed, mean number of alleles (MNA) per locus and data format translation were calculated using Microsatellite Toolkit 3.1 (<http://animalgenomics.ucd.ie/sdepark/ms-toolkit>).

Polymorphic Information Content (PIC) was calculated for each microsatellite locus (Botstein *et al.*, 1980). The exact test for Hardy-Weinberg equilibrium across all loci and subpopulations were performed with GENEPOP (v. 1.2) with their standard deviations using Markov-Chain noted by Raymond and Rousset (1995).

Fixation coefficients ( $F_{st}$ ,  $F_{is}$  and  $F_{it}$ ) per pair of populations and their statistical significance were computed using the FSTAT program (Goudet, 2001). A hierarchical analysis was performed to examine the population differentiation using analysis of molecular variance (AMOVA) in the

Table 2: Information on 14 microsatellite loci used in the study

Locus	No. of alleles	MNA	Ho	PIC	Fit	Fst	Fis
AHT4	10	9.8	0.752	0.842	0.129**	0.020**	0.112**
ASB17	15	12.2	0.694	0.873	0.212**	0.021**	0.195**
ASB23	13	9.8	0.615	0.803	0.257**	0.019**	0.243**
HTG7	5	4.8	0.565	0.675	0.223**	0.014	0.212**
HMS2	10	8.6	0.806	0.780	0.005	0.007	-0.002
HTG6	8	7.4	0.602	0.707	0.209**	0.025**	0.189**
HMS6	8	6.8	0.744	0.753	0.051	0.027**	0.024
HTG10	10	9.6	0.649	0.813	0.218**	0.023**	0.199**
LEX33	11	8.2	0.773	0.781	0.027	0.021**	0.007
ASB2	12	10.2	0.782	0.824	0.085**	0.022**	0.063*
VHL20	9	8.2	0.738	0.834	0.140**	0.017**	0.125**
HMS3	8	7.2	0.674	0.783	0.164**	0.027**	0.141**
HMS7	8	7.4	0.740	0.722	0.002	0.016**	-0.015
HTG4	6	5.4	0.603	0.632	0.095**	0.051**	0.046
Mean <sup>1</sup>	9.5	8.3	0.695	0.773	0.130**	0.021**	0.111**
Mean <sup>2</sup>		—			0.112**	0.013**	0.100**

\*p<0.05, \*\*p<0.01. <sup>1</sup>For all populations. <sup>2</sup>For the 3 Tibetan Horse populations

Table 3: Diversity information on Tibetan horse populations, Mongolian horse and Ningqiang Pony

Population	MNA	Ne	Ho	He	PIC
Tibetan horse	13.1	5.01	0.710	0.796	0.752
Qamdo	12.9	5.12	0.762	0.786	0.754
Nagqu	10.3	4.87	0.694	0.787	0.745
Xigaze	16.1	5.06	0.676	0.792	0.759
Mongolian horse	12.3	5.35	0.692	0.807	0.773
Ningqiang Pony	9.7	4.40	0.650	0.768	0.726

PA<sup>1</sup>: No. of private alleles

Arlequin (Schneider *et al.*, 1997). Principal Component Analysis (PCA) with gene frequency data was performed using the MVSP version 3.1 program (available at <http://www.kovcomp.co.uk/mvsp/>).

Nei's genetic distance  $D_A$  (Nei *et al.*, 1983) and Nei's standard genetic distance  $D_S$  (Nei, 1972) between breeds were estimated and used to construct the Unweighted Pair Group Method with Arithmetic (UPGMA) mean dendrograms using DISPAN software (Ota, 1993).

## RESULTS

**Genetic variability:** Totally, 133 alleles were detected across the 14 microsatellite loci (Table 2). Number of alleles per locus varies from 5 (HTG7) to 15 (ASB17). The MNA across 14 loci was 8.3 for the five horse populations. The observed heterozygosity across all loci in the total samples was 0.695 and ranged from 0.565 (HTG7) to 0.806 (HMS2). More information related to diversity across the microsatellite loci is given in Table 2.

The Mean Number of Alleles (MNA) in Tibetan Horse was 13.1, varied between 10.3 in Nagqu Horse and 16.1 in Xigaze Horse (Table 3), which might be explained by the variation in the sample sizes (The sample number for Nagqu Horse and Xigaze Horse were 39 and 61, respectively).

**F-statistic:** The Fst values for each locus were very close and were shown with Fit and Fis values in Table 2. Highly significant (p<0.01) genetic differentiation (Fst) was detected for all loci. On

Table 4: Pairwise population differentiation *Fst* estimates (below the diagonal) between horse breeds and Nei's standard genetic distance (Ds, above the diagonal) among 5 Chinese local horse population

	Qamdo	Nagqu	Xigaze	Mongolian horse	Ningqiang Pony
Qamdo		0.071	0.039	0.028	0.071
Nagqu	0.018**		0.064	0.064	0.121
Xigaze	0.010**	0.015**		0.026	0.080
Mongolian horse	0.006**	0.014**	0.005**		0.066
Ningqiang Pony	0.018**	0.030**	0.020**	0.017**	

\*p<0.05, \*\*p<0.01

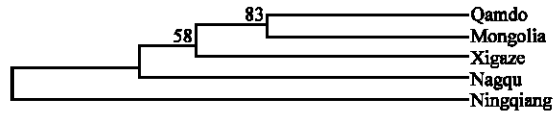


Fig. 2: Dendrogram of relationships among 5 Chinese local horse populations using Ds genetic distance and the neighbor-joining method in Dispan programme

average, only 2.1% of the variation accounted for total population differentiation and breeds had a 11.1% (p<0.01) deficit of heterozygotes, whereas the total population had a 13% (p<0.01) deficit of heterozygotes. *Fst* values were showed in Table 4 when breeds were considered in couples. The values of *Fst* between Tibetan Horse ranged from 1% for the Qamdo-Xigaze pair to 1.8% for the Nagqu-Qamdo pair. The highest *Fst* was detected between Nagqu and the Ningqiang Pony (3%).

**Relationships among horse populations:** Values above the diagonal in Table 4 represent the Ds genetic distance, using 14 microsatellite loci, ranged between 0.026 and 0.121 for the five breeds, the results of which were also very similar to ones of *Fst*. The low genetic distances among the five breeds indicated a close relationship among them.

$D_A$  and Ds distance matrices were used to build phylogenetic trees with UPGMA and NJ methods. The UPGMA dendrogram (Fig. 2) shows the relationship between horse populations and the reliability of the obtained tree was examined by 1000 bootstrap replicates. The most robust features of the topology were the cluster (83% support) formed by Qamdo and Mongolian Horse, then the cluster came together with Xigaze. The remaining breeds Ningqiang Pony and Nagqu had separate clusters.

**DISCUSSION**

All of the microsatellite loci genotyped were highly informative in Tibetan Horse, Mongolian Horse and Ningqiang Pony (PIC>0.6). The average observed heterozygosity in Tibetan Horse was 0.71, which was close with those (0.635-0.707) of Lipizzan horse (Achmann *et al.*, 2004). The average PIC and expected heterozygosity for 14 loci in Mongolian Horse was 0.773 and 0.807, respectively, which was little different from the results (0.761 and 0.809) in Korea horse and Alien horse using the same microsatellite markers (Cho, 2006). Cho reported that Mongolian horse retained the largest amount of genetic variation in all the populations studied. Japanese reported Japanese native horse had lower diversity than Mongolian population and all of the native population were derived from Mongolian horse (Kakoi *et al.*, 2007). Whereas, in the present study, the observed heterozygosity in Mongolian Horse (0.692) was lower than Cho's report (0.833), but

higher than Li's (0.482). Tibetan Horse had higher genetic diversity than Mongolian Horse and Ningqiang Pony, in which Qamdo population was highest in observed heterozygosity ( $H_o = 0.762$ ). It can be imagined that Mongolian native horse had decline in genetic diversity because of the policy in pasturing area caused the rapidly decline of Mongolian Horse, which restrains the populations of horse and domestic animals to protect the environment of grass land in Mongolia. The lowest genetic diversity of Ningqiang Pony was in agreement with its endangered status described (Ma *et al.*, 2002; Zhang *et al.*, 2008a; Sun, 2008).

Although, abundant genetic variation was detected, the population differentiation in the studied local horses was not significant. The value of 2.1% of the genetic variation was lower than those from other genetic diversity studies, e.g. 10% for Polish endangered Bilgoraj horses and two common horse breeds (Zabek *et al.*, 2005) and 8% of the Spanish Celtic horse breeds (Canon *et al.*, 2000), but was almost the same with the  $F_{st}(0.024)$  reported in Chinese (Ling *et al.*, 2009). It suggested that our results were consistent with the researches on other Chinese indigenous horse breeds. However, the  $F_{is}(0.111)$  in the study was much higher than the  $F_{is}$  (0.021) in Ling's. It suggested that the horse breeds in the study has inbreeding in an extent.

The corresponding UPGMA trees clustered the Mongolian native horse together with the Qamdo, the close association between Qamdo and Mongolian native horse support Northern influence on Tibetan Horse. The history records could be helpful to clarify that. Genetic Contribution of Mongolian Horse to Tibetan horse populations is in accordance with Mongolian army's western aggressive immigration route and the direction of genetic contribution is compatible with human genetic studies (Chen *et al.*, 2006). Chen reported though the contemporary Tibetans inhabit the Southwest China, Tibetans were genetically closer to Northern Han Chinese, Mongolian Chinese rather than to Southern Han Chinese.

Qamdo and Xigaze are both agricultural areas of the Tibetan Horse with comparatively lower altitude, round about 3500 m, where Tibetan Horse may be extensively influenced by Mongolian Horse. Therefore, their higher genetic relationship with Mongolian Horse is not surprising. As average altitude is about 4500 m in Nagqu prefecture, Nagqu Horse was hardly affected by any other horse populations such as Mongolian Horse, which could not adapt to the specific tough ecological condition. That may be an important reason why Nagqu horse is distinct separate with the other two Tibetan Horse populations and referenced breeds.

The results of the present study showed far genetic distance between Tibetan Horse and Ningqiang Pony which represented Southwest Type of the indigenous horses in China. The results are in accordance with previous description by Xie (1986) that Tibetan Horse is an ancient unique horse breed which could not be classified into Southwest Horse.

## ACKNOWLEDGMENTS

We acknowledge the assistance of the following people with sample collection: Y. Chamba, H. Zhang, S. Lian, L. Deng. We are also grateful for the encouragement and valuable statistical suggestions provided by Y. Zhang and W. Liu

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