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Evaluation of Genetic Diversity and Population Structure of Five Chinese Indigenous Donkey Breeds Using Microsatellite Markers

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ABSTRACT

Di R., Liu Q.Y., Xie F., Hu W.P., Wang X.-Y., Cao X.-H., Pan Z.-Y., Chen G.-H., Chu M.-X. (2017): **Evaluation of genetic diversity and population structure of five Chinese indigenous donkey breeds using microsatellite markers**. Czech J. Anim. Sci., 62, 219–225.

China had the largest population of raising donkeys in the world, however the number of Chinese indigenous donkey decreased dramatically due to the increase of agriculture mechanization in the last century. The species has still been important in China because of its edible and medical value, therefore the survey on its genetic diversity in China is necessary for its conservation and utilization. In this study, 15 microsatellite markers were used to evaluate genetic diversity and population structure of five Chinese indigenous donkey breeds. The mean values of expected heterozygosity, allelic richness, and total number of alleles for all the tested Chinese donkeys were 0.70, 6.04, and 6.28 respectively, suggesting that the genetic diversity of Chinese indigenous donkeys is rich. The Bayesian analysis and principal component analysis plot yielded the same clustering result, which revealed that Guanzhong donkey was the most differentiated breed in all detected samples, and Jinnan (JN) and Guangling (GL) were genetically closed together. Additionally, our results indicated that the heterozygote deficit was severe in two Chinese indigenous donkey breeds (GL and JN), and it warned us that animal conservation activities on this species should be considered carefully in near future.

Keywords: donkey; genetic richness; genetic differentiation; heterozygote deficit

Ancient DNA study implied that domestic donkey was introduced into China 2000 years ago (Han et al. 2014). China had the largest population of raising donkeys in the world (Xie 1987). In the last century, the donkey was widely used as an important agricultural labour of draft and burden in China. However since the increase of agriculture mechanization in last four decades, the number of donkeys as draught animals has decreased dramatically. Recently, the species has become an economically important animal in China because donkey meat has been found to have

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high nutritional value and the Colla Corii Asini (donkey-hide glue) is popularly used as a traditional Chinese medicine. With the recent severe reduction in the number of indigenous donkey, the investigations of Chinese donkey breeds genetic diversity and structure are necessary for further conservation and utilization of this species.

To date, the genetic diversity of indigenous donkey breeds in Spain (Aranguren-Mendez et al. 2001; Aranguren-Mendez et al. 2002), Croatia (Ivankovic et al. 2002), Italy (Bordonaro et al. 2012; Colli et al. 2013; Matassino et al. 2014), America (Jordana et al. 2015), northeast Africa, the Near East and the Arabian Peninsula (Rosenbom et al. 2015) has been evaluated using microsatellite markers, and these results of evaluation are crucial for making further conservation strategy. On the other hand, the origin of the species and evolutionary relationship between donkey breeds were analyzed using the mitochondrial DNA markers (Ivankovic et al. 2002; Lopez Lopez et al. 2005; Chen et al. 2006; Zhang et al. 2010; Han et al. 2014; Perez-Pardal et al. 2014), and these results hinted that the modern donkey (including Chinese donkey) has an African maternal origin. In this study, the genetic diversity and structure of five Chinese donkey breeds were assessed using 15 microsatellite markers in order to provide important implications for further conservation and utilization of Chinese donkeys.

MATERIAL AND METHODS

Sample collection and DNA extraction. Blood samples (10 ml per donkey) were collected from 35 Guanzhong (GZ) donkeys (Fufeng County, Shaanxi Province, P.R. China), 36 Jinnan (JN) donkeys (Xia County, Shanxi Province, P.R. China), 27 Guangling (GL) donkeys (Shouyang County, Shanxi Province, P.R. China), 25 Dezhou (DZ) donkeys (Wudi County, Shandong Province, P.R. China), and 30 Huabei (HB) donkeys (Xushui County, Hebei Province, P.R. China). Genomic DNA was recovered from blood using a standard phenol-chloroform extraction method, then dissolved in TE buffer (10 mmol/l Tris–HCl (pH 8.0), 1 mmol/l EDTA (pH 8.0)) and kept at –20°C.

Microsatellite loci and genotyping. Fifteen microsatellite loci were screened from the set recommended by ISAG/FAO and previous reports (Eggleston-Stott et al. 1997; Meyer et al. 1997; Swinburne et al. 1997; Tallmadge et al. 1999a, b). The relative information of these loci was shown in Table 1. Polymerase chain reaction (PCR) was performed in 12 µl reaction volumes containing 50 ng of genomic DNA, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 0.025 µM of each primer, 1.25 units of *Taq* polymerase, and 1× magnesium-free PCR buffer (TaKaRa, Japan) using Mastercycler® 5333 (Eppendorf AG, Germany). The cycling parameters were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing temperature (Table 1) for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. Genotyping was conducted using an ABI 3730xl DNA analyzer and GeneMapper Version 3.7 software (Applied Biosystems, USA). The third-order least squares method was adopted for allele size determination (Mburu et al. 2003). Data was available from the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.m5t70).

Statistical analysis. Expected heterozygosity (*He*) and observed heterozygosity (*Ho*) for each breed and each locus were calculated using POPGENE Version 1.31 (http://www.ualberta.ca/~fyeh). Total number of alleles (TNA) for each locus was also obtained using the same software. Nei's standard genetic distance (D_S) (Nei 1972) and Nei's genetic distance (D_A) (Nei et al. 1983) were calculated by the DISPAN software package. The *F*-statistic values (F_{ST}, F_{IT}, and F_{IS}) and the allelic richness (AR) were estimated by FSTAT Version 2.9.3 (Goudet 2001). Fisher's exact test was performed to test possible significant departures from the Hardy–Weinberg (HW) proportions using GENEPOP Version 3.4 (Raymond and Rousset 1995). *P*-values of heterozygote deficit and excess for each locus were obtained simultaneously.

The population genetic structure was revealed with STRUCTURE 2.1 software (Pritchard et al. 2000). Six independent runs of the Gibbs sampler for $2 \leq K \leq 5$ were performed using a burn-in of 10^6 followed by 10^6 Markov chain Monte Carlo iterations. We determined the optimal *K* value using the method of Evanno et al. (2005), who proposed that ΔK (an *ad hoc* quantity related to the second order rate of change of the log probability of data) acts as a better predictor of the real number of clusters compared with the highest *Ln*. Finally, the graphical displays of the population structure were generated using the DISTRUCT program (Rosenberg 2004). Additionally, based on allele frequencies, principal component analysis (PCA) was performed using MVSP 3.1 program (http://www.kovcomp.com).

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Table 1. Primer information and basic genetic parameters for five Chinese donkey breeds at 15 microsatellite loci

Locus	Chr	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Tm (°C)	At	Rt	F_{ST}	F_{IT}	F_{IS}
AHT21	1	TCCAAGTTGCTGAATGGATC	ACGGCCTGATTCTCTCTTTG	60	9	7.564	0.047***	0.125**	0.082*
UCDEQ437	3	CTGTTCTGGGCAGGCTTCTCTA	TTGCTGGCTTGGCTGGTC	60	10	9.096	0.025***	0.423***	0.230***
HTG07	4	CCTGAAGCAGAACATCCCTCCTTG	ATAAAGTGTCTGGGCAGAGCTGCT	58	12	9.909	0.131***	0.245***	0.130**
COR070	6	CATCTGTTCCGTGGCATTA	TTCAGGTGTGGGTTTTGAATC	58	8	5.174	0.019***	0.055	0.037
COR095	7	TACCTCTGGTGGTGATGCTT	CCCACACTTACTCCCATCAC	58	10	6.945	0.293***	0.511***	0.326***
HMS03	9	CCAACTCTTTGTCACATAACAAGA	CCATCCTCACTTTTTCACTTTGTT	58	7	4.808	0.011**	0.008	–0.004
HMS02	10	ACGGTGGCAACTGCCAAGGAAG	CTTGCAGTCGAATGTGTATTAAATG	58	12	9.716	0.006	0.586***	0.584***
HTG06	15	CCTGCTTGGAGGCTGTGATAAGAT	GTTCACTGAATGTCAAATTCTGCT	58	4	3.995	0.036***	–0.093	–0.134
UCDEQ505	16	ATCACTCTCTTGTTGAGATAAC	GGGATTTCCTTCTTTCTC	55	8	6.531	0.054***	0.121**	0.073
UM011	20	TGAAAGTAGAAAGGGATGTGG	TCTCAGAGCAGAAGTCCCTG	56	11	8.717	0.074***	0.128***	0.058
HTG10	21	CAATTCCCGCCCCACCCCCGGCA	TTTTTATTCTGATCTGTCACATTT	54	10	6.883	0.021	0.028	0.009
AHT04	24	AACCGCCTGAGCAAGGAAGT	CCCAGAGAGTTTACCCT	58	10	6.771	0.026***	0.042	0.017
COR071	26	CTTGGGCTACAACAGGGAATA	CTGCTATTTCAAACACTTGGA	56	8	5.690	0.041***	0.273***	0.240***
UCDEQ425	28	AGCTGCCTCGTTAATTCA	CTCATGTCCGCTTGTCTC	60	6	5.559	0.180***	0.722***	0.660***
COR082	29	GCTTTTGTTTCTCAATCCTAGC	TGAAGTCAAATCCCTGCTTC	58	7	6.916	0.035**	0.6***	0.586***
Total					132	7.564	0.074***	0.199***	0.258***

Chr = chromosome assignment, Tm = annealing temperature, At = total number of alleles per locus, Rt = allelic richness over all samples, F_{ST} = fixation index resulting from comparing subpopulations to the total population, F_{IT} = fixation indices of total population, F_{IS} = fixation indices of subpopulation

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

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RESULTS

HWE test and polymorphism of microsatellite markers. All of the microsatellites used in this study were amplified and were polymorphic in the domestic donkey breeds. Generally, the allelic polymorphism in the 15 loci was high for the five donkey breeds. A total of 132 alleles were detected at the 15 loci. The mean number of alleles across all loci was 8.8, and for single locus the number of alleles (At) ranged from 4 (*HTG06*) to 12 (*HTG07* and *HMS02*). The mean AR across the 15 loci in our Chinese donkey breeds was 6.952, and for single locus the AR over all samples (Rt) varied between 4.808 (*HMS03*) and 9.909 (*HTG07*).

The lack of linkage between loci was verified using the Markov chain method. In all the breed–locus combinations tested, significant deviations ($P < 0.05$) from HW proportions were observed in 14 (18.67%) out of 75 breed–locus combinations (Supplementary Table S1). The GZ showed the maximum number of loci in disequilibrium (5 loci), followed by GL (3 loci). However, for either a single locus across all breeds or a single breed across all loci, there was no significant departure from the HW proportion ($P > 0.05$).

Genetic diversity of five donkey breeds. A summary of the polymorphisms for all tested donkey breeds was presented in Table 2. For five donkey breeds, the highest *He* (0.73) was found in HB, and the lowest *He* (0.68) was found in GZ. TNA ranged from 5.93 (DZ) to 6.80 (JN). The value of AR was the highest in JN (AR = 6.36) and the lowest in GZ (AR = 5.78). The mean values of *He*, AR, and TNA for all the tested Chinese indigenous donkeys were 0.70, 6.04, and 6.28, respectively.

Table 2. Information on genetic diversity for 15 microsatellite loci in five Chinese indigenous donkey breeds

Parameter	Breed					
	GZ	JN	GL	DZ	HB	mean
He	0.68	0.71	0.69	0.69	0.73	0.70
Ho	0.55	0.54	0.55	0.57	0.61	0.56
TNA	6.07	6.80	6.00	5.93	6.60	6.28
AR	5.78	6.36	5.87	5.86	6.32	6.04
F_{IS}	0.199**	0.220**	0.225**	0.172**	0.172**	

GZ = Guanzhong donkey, JN = Jinnan donkey, GL = Guangling donkey, DZ = Dezhou donkey, HB = Huabei donkey, *He* = expected heterozygosity, *Ho* = observed heterozygosity, TNA = total number of alleles, AR = allelic richness, F_{IS} = fixation indices of subpopulation and significant levels of deficit or excess in heterozygotes (deficit: $^{*}P < 0.05$, $^{**}P < 0.01$; excess: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$)

Table 3. Nei's genetic distance D_A (above the diagonal) and Nei's standard genetic distance D_S (below the diagonal) between five Chinese donkey breeds

	GZ	JN	GL	DZ	HB
GZ		0.1734	0.1617	0.1885	0.1695
JN	0.2638		0.0936	0.1184	0.1469
GL	0.2741	0.0905		0.1350	0.1644
DZ	0.2608	0.1305	0.1928		0.1626
HB	0.2982	0.2277	0.3007	0.2487	

GZ = Guanzhong donkey, JN = Jinnan donkey, GL = Guangling donkey, DZ = Dezhou donkey, HB = Huabei donkey

Population structure of five donkey breeds. As shown in Table 1, the inbreeding coefficients (F_{IS}) for 13 loci were positive, and their values in 7 loci reached an extremely significant level ($P < 0.001$). For five donkey breeds, all F_{IS} values were positive (Table 1). Of them, GL and JN had the highest F_{IS} values (0.225 and 0.220, respectively) and most loci (both are 6) exhibited heterozygotes deficit at a significant level. It is worth noting that the differences between the *He* and *Ho* of the two breeds (GL and JN) were also the largest of the five donkey breeds studied.

The total F_{ST} for Chinese five donkey breeds was 0.074, suggesting that 92.6% of the total genetic variation of all tested donkeys resulted from genetic differentiation within breed and 7.4% existed

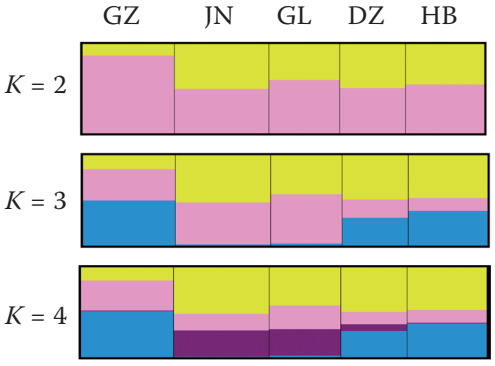

Figure 1. Population structures of five Chinese indigenous donkey breeds displayed with population **Q** matrices when assumed cluster $K = 2$, 3, and 4
GZ = Guanzhong donkey, JN = Jinnan donkey, GL = Guangling donkey, DZ = Dezhou donkey, HB = Huabei donkey

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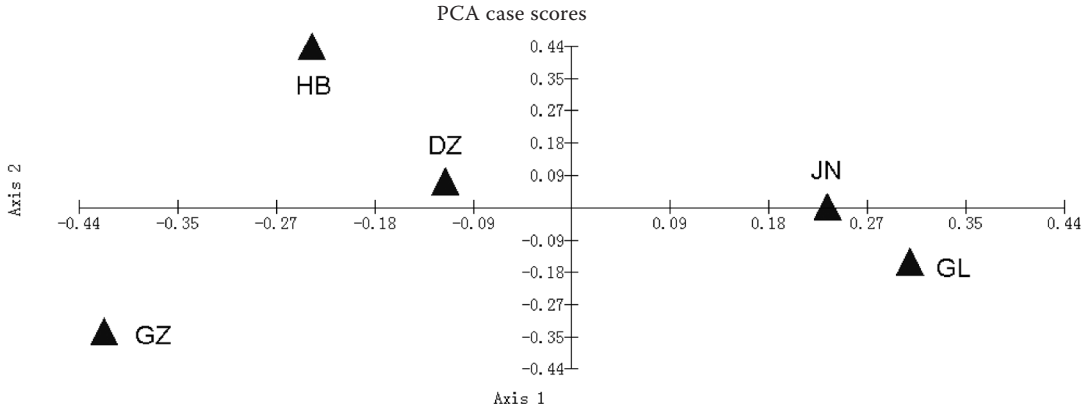

Figure 2. Principal component analysis (PCA) of five Chinese indigenous donkey breeds based on allele frequency
GZ = Guanzhong donkey, JN = Jinnan donkey, GL = Guangling donkey, DZ = Dezhou donkey, HB = Huabei donkey

between breeds. A significant genetic differentiation (F_{ST}) ($P < 0.001$) was detected in each pair of donkey breeds ranging from 0.0423 (JN/GL) to 0.1060 (GL/GZ). The results of genetic distances (Table 3) showed that the greatest distance existed in GZ–DZ (D_A) and GL–HB (D_S) pairs, and the lowest distance was observed between JN and GL.

Using STRUCTURE 2.1, we firstly determined an optimum K value at 2 following the method of Evanno. Combining the results for K = 2, 3, and 4, we can see that GZ was different from the other breeds and the structures of JN and GL were the most similar (Figure 1). To verify the Bayesian clustering result, we tried the other two clustering methods. The PCA result for the five breeds was shown in Figure 2. The results of the two clustering methods indicated that JN and GL were evolutionarily closed together, then clustered with DZ, however GZ breed was the most differentiated breed of all the samples detected.

DISCUSSION

Chinese donkey breeds analyzed in this study exhibited a relatively rich genetic diversity. As far as mean *He* of all loci be concerned, Chinese donkey breeds were similar to other highly diversified breeds in the Near East, northeast Africa (Rosenbom et al. 2015), Spain (Aranguren-Mendez et al. 2001), and Croatia (Ivankovic et al. 2002), and more diversified than Italian (Bordonaro et al. 2012; Colli et al. 2013; Matassino et al. 2014) and American (Jordana et al. 2015) donkeys. For *He* of the same single locus in comparable studies, Chinese donkeys were also similar to or slightly higher than European (Aranguren-Mendez et al. 2001; Jordana et al. 2001; Ivankovic et al. 2002; Bordonaro et al. 2012; Colli et al. 2013) and American (Jordana et al. 2015) donkeys. Those results were consistent with reports of previous mtDNA data on Chinese donkey breeds (Lei et al. 2007; Zhang et al. 2010).

GL and JN had the highest F_{IS} values, the most loci with heterozygotes deficit, and the biggest difference between the expected heterozygosity and observed heterozygosity among the five tested breeds. These results together suggested a predominance of mating between close relatives or small effective population sizes in these two breeds. During the last four decades, Chinese indigenous donkey breeds suffered from a severe reduction in population size along with the enhancement of agriculture mechanization. Meanwhile the artificial insemination and selecting for the excellent breeding stock were popular during the donkey breeding. All these practices led to the heterozygote deficiencies in Chinese indigenous donkeys, and evoked the necessity of careful selecting a proper strategy on further conservation of the resource. Immediate measure on the species should be considered carefully, e.g. the increase in the number of breeding jackasses and mares, avoiding selection of animals with a close genetic relationship.

Population structure analysis and PCA results provided the same picture, which revealed that GZ is the most differentiated breed of all detected breeds. JN and GL were the nearest according to the genetic relationship. The clustering results were partly consistent with their geographical

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distribution. JN and GL were distributed in the same province (Shanxi province), so the geographic distance between the sample locations was comparatively smaller in this study. GZ occurred in Shaanxi province of northwest China, far away from the other breeds. However, the geographical structure was not obvious for all the Chinese indigenous donkeys.

The global F_{ST} estimate (7.4%) of Chinese donkeys detected in this study was similar to the value of American donkeys (Jordana et al. 2015) but lower than that of donkeys in the Near East and northeast Africa (Rosenbom et al. 2015). Previous reports of Beja-Pereira et al. (2004), Chen et al. (2006), Lei et al. (2007), Zhang et al. (2010), and Perez-Pardal et al. (2014) indicated a weak phylogeographic structure of indigenous donkey breeds by mtDNA studies. Using pedigree information, Gutierrez et al. (2005) also suggested a weak population structure of indigenous Catalonian donkey breeds. The loss of donkey phylogeographic structure may mainly result from a very quick spread of the species after domestication, due to its movability and serving for long-distance transport (Beja-Pereira et al. 2004).

CONCLUSION

In summary, our results suggested the relatively high genetic diversity of Chinese indigenous donkeys and brought an insight in the structure of the analyzed populations. The heterozygote deficit was severe in two Chinese indigenous donkey breeds, which warned us that animal conservation activities on the species should be considered carefully in near future.

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