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High polymorphism at microsatellite loci in the Chinese donkey

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ABSTRACT. To reveal the genetic diversity and phylogenetic relationships between Chinese donkey breeds, 415 individuals representing ten breeds were investigated using ten microsatellite markers. The observed number of alleles, mean effective number of alleles (N_E), mean expected heterozygosity (H_E), and polymorphic information content (PIC) of each breed and polymorphic locus were analyzed. The results showed that seven (HTG7, HTG10, AHT4, HTG6, HMS6, HMS3, and HMS7) of ten microsatellite loci were polymorphic. The mean PIC, H_E, and N_E of seven polymorphic loci for the ten donkey breeds were 0.7679, 0.8072, and 6.0275, respectively. These results suggest that domestic Chinese donkey breeds possess higher levels of genetic diversity and heterozygosity than foreign donkeys. A neighbor-joining tree based on Nei's standard genetic distance showed that there was close genetic distance among Xinjiang, Qingyang, Xiji,

and Guanzhong donkey breeds. In addition, Mongolia and Dezhou donkey breeds were placed in the same category. The phylogenetic tree revealed that the genetic relationships between Chinese donkey breeds are consistent with their geographic distribution and breeding history.

Key words: Chinese donkey; Microsatellite; Polymorphism; Phylogenetic relationship

INTRODUCTION

China has a long history of raising donkeys. There are more than 9 million donkeys distributed widely in China, accounting for about 22% of the total world population (Hou and Hou, 2002). Based on their body size, 24 different donkey breeds in China can be classified into three types: large, middle, and small (Yang and Hong, 1989). However, since the 1980s, the donkey population has been largely reduced and some breeds are at risk of extinction due to agricultural mechanization and economic development in China (Ma et al., 2002). Therefore, the need to conserve this valuable genetic resource is urgent, and assessing the genetic diversity and relationships between and within donkey breeds is a critical step in this process.

Molecular markers, especially microsatellites, are effective tools used to evaluate genetic diversity and relationships within and between animal breeds. The identification of polymorphic markers will allow the levels of genetic variability and degree of inbreeding to be estimated, as well as parentage verification and the identification of the most heterozygous individuals in the population (Jordana et al., 2001). Jordana et al. (2001) found that microsatellite loci are effective at revealing genetic variation among donkey breeds in the Catalonian region. Using 15 microsatellite markers to analyze the hierarchical genetic structure of five endangered Spanish donkey breeds, Aranguren-Méndez et al. (2002) revealed that four breeds with black coats from northern Spain form a closed cluster and supported the hypothesis of a common ancestral past from *Equus asinus europeus*. Using eight microsatellites, Ivankovic et al. (2002) revealed relatively high levels of heterozygosity in three donkey populations in the coastal region of Croatia. Previous studies have also found high levels of diversity within and between several breeds of Chinese donkey using microsatellites (Xie, 2004; Zhu and Su, 2011); however, the findings of these studies were not representative, as they did not incorporate all three types of donkeys.

This study aimed to investigate the degree of genetic diversity and relationships among 10 local donkey breeds, which represent all three types of Chinese domestic donkey, using 10 microsatellite markers. These 10 microsatellites have been shown to be effective markers in the study of genetic diversity of donkeys in previous studies (Jordana et al., 2001; Aranguren-Méndez et al., 2002; Ivankovic et al., 2002; Xie, 2004; Zhu and Su, 2011).

MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 415 individuals were collected from 10 Chinese indigenous donkey breeds (Table 1), including three types of donkey: large (Guanzhong, Dezhou), medium (Qingyang, Biyang, and Jiami), and small (Mongolia, Gunsha, Xinjiang, Taihang, and Xiji). These breeds

are generally distributed along the Yellow River region and represent the major genetic resources of Chinese donkey. Genomic DNA was isolated from peripheral blood using a standard phenol-chloroform protocol (Green and Sambrook, 2012).

Table 1. Location and sample size of 10 Chinese domestic donkey breeds.

Breed	Code	Sample size	Location	Type
Dezhou	DZ	35	Dezhou city, Shandong Province	Large
Guanzhong	GZ	39	Fufeng county, Shaanxi Province	Large
Qingyang	QY	36	Qingyang city, Gansu Province	Medium
Jiami	JM	36	Mizi county, Shaanxi Province	Medium
Biyang	BY	26	Biyang county, Henan Province	Medium
Taihang	TH	38	Linzhou county, Henan Province	Small
Gunsha	GS	37	Yulin city, Shaanxi Province	Small
Mongolia	MG	97	Chifeng city, Inner Mongolia region	Small
Xinjiang	XJ	39	Yining county, Xinjiang region	Small
Xiji	GY	32	Guyuan county, Ningxia region	Small

Microsatellite marker genotyping

Ten microsatellite markers were used for genotyping (Table 2). PCR amplifications were performed in 25-μL reactions containing 40 ng template DNA, 10 pM each primer, 12.5 μL 2X PCR mix buffer (0.75 U *Taq* DNA polymerase, 2X PCR buffer, 37.5 μM $MgCl_2$, and 5 μM dNTPs) with the following conditions: initial denaturation at 95°C for 5 min, 36 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperatures (Table 2), and elongation at 72°C for 45 s, final extension at 72°C for 10 min, following which samples were held at 4°C. Afterward, 3-5 μL PCR products were electrophoresed in 10% native polyacrylamide gel at 120 V for 7-10 h. The gels were stained with silver nitrate and the fragment sizes were determined using a Kodak Digital Science ID Image Analysis Software System.

Table 2. Primer sequences and annealing temperatures.

Microsatellite loci	Primer sequences (5'→3')	Annealing temperature (°C)	References
AHT4	F: AAC CGC CTG AGC AAG GAA GT R: GCT CCC AGA GAG TTT ACC CT	64.0	Binns et al. (1995)
HTG6	F: CCT GCT TGG AGG CTG TGA TAA GAT R: GTT CAC TGA ATG TCA AAT TCT GCT	60.0	Ellegren et al. (1992)
HTG7	F: CCT GAA GCA GAA CAT CCC TCC TTG R: ATA AAG TGT CTG GGC AGA GCT GCT	65.5	Marklund et al. (1994)
HTG10	F: CAA TTC CCG CCC CAC CCC CGG CA R: TTT TTA TTC TGA TCT GTC ACA TT	58.8	Marklund et al. (1994)
HMS3	F: CCA ACT CTT TGT CAC ATA ACA AGA R: CCA TCC TCA CTT TTT CAC TTT GTT	58.0	Guérin et al. (1994)
HMS6	F: CAA GCT GCC AGT ATT CAA CCA TTG R: CTC CAT CTT GTG AAG TGT AAC TCA	64.3	Guérin et al. (1994)
HMS7	F: CAG GAAACT CAT GTT GAT ACC ATC R: TGT TGT TGA AAC ATA CCT TGA CTG T	57.0	Guérin et al. (1994)
HMS1	F: CAT CAC TCT TCA TGT CTG CTT GG R: TTG ACA TAA ATG CTT ATC CTA TGG C	56.0	Guérin et al. (1994)
HTG4	F:CTATCTCAGTCTTGATTGCAGGAC R:CTCCCTCCCTCCCTCTGTTCTC	60.0	Ellegren et al. (1992)
HMS5	F: TAG TGT ATC CGT CAG AGT TCA AA R: GCA AGG AAG TCA GAC TCC TGG A	62.0	Guérin et al. (1994)

Data analysis

Allele numbers, frequencies, mean expected heterozygosity (H_E) and mean effective number of alleles (N_E) for each marker were obtained in each breed using the POPGENE 1.32

software. Polymorphic information content (PIC) for each locus was calculated based on the research of Botstein et al. (1980).

Standard genetic distances (*D*s) and Nei's standard genetic distances (D_A) were obtained based on allele frequencies, and a neighbor-joining tree based on D_A was constructed, which was evaluated by bootstrap resampling of each locus (Nei, 1972). Furthermore, total population heterozygosity (H_T), subpopulation heterozygosity (H_S), and an estimator of the degree of genetic differentiation ($G_{ST} = 1 - H_S/H_T$) were also obtained. All calculations were carried out using DISPAN package (Ota, 1993).

RESULTS

Genetic variability of 10 donkey breeds at 10 microsatellite loci

Among the 10 microsatellite loci studied, seven (HTG7, HTG10, AHT4, HTG6, HMS6, HMS3, and HMS7) were polymorphic, while the remaining loci including (HMS1, HTG4, and HMS5) were monomorphic. As shown in Table 3, the N_E for 10 Chinese donkey breeds was 6.0275, ranging from 2.1855 [Dezhou donkey (DZ)] to 10.9050 [Jiami donkey (JM)]. H_E for the whole population was 0.8072, ranging from 0.5503 (DZ) to 0.9217 (JM). The mean PIC for the seven polymorphic loci was 0.7679, ranging from 0.4561 (DZ) to 0.8893 [Xinjiang donkey (XJ)].

Table 3. Genetic index of seven microsatellite loci in 10 Chinese donkey breeds.

Locus	Genetic index	DZ	GY	GZ	GS	JM	BY	QY	TH	XJ	MG
AHT4	PIC	0.8587	0.8159	0.8784	0.8180	0.8561	0.8429	0.8792	0.8282	0.8719	0.8838
	H_E	0.8865	0.8488	0.8940	0.8503	0.9217	0.8768	0.9038	0.8572	0.8951	0.8983
	N_E	7.9288	6.0287	8.4941	6.1653	10.905	7.0675	9.0619	6.4899	8.5697	9.3909
HTG6	PIC	0.8149	0.8041	0.8095	0.8246	0.7541	0.8018	0.7986	0.8490	0.8530	0.8124
	H_E	0.8480	0.8407	0.8422	0.8556	0.7975	0.8425	0.8323	0.8760	0.8797	0.8382
	N_E	6.0945	5.7692	5.9298	6.4000	4.6756	5.6882	5.5577	7.3602	7.5635	6.0125
HTG10	PIC	0.8720	0.8525	0.8559	0.8453	0.7992	0.8075	0.8618	0.8210	0.8893	0.8756
	H_E	0.8957	0.8810	0.8808	0.8732	0.8336	0.8478	0.8889	0.8502	0.9104	0.8909
	N_E	8.5366	7.5294	7.6625	7.2000	5.5846	5.7313	8.0000	6.2108	9.8136	8.7807
HMS3	PIC	0.8803	0.8093	0.8549	0.7530	0.8087	0.7946	0.8354	0.8186	0.8032	0.8818
	H_E	0.9039	0.8390	0.8807	0.7953	0.8410	0.8338	0.8652	0.8920	0.8364	0.8965
	N_E	9.1383	5.7143	7.5851	4.5981	5.7926	5.4256	6.7203	8.2571	5.6977	9.2305
HMS7	PIC	0.4561	0.5061	0.4793	0.5204	0.4786	0.5604	0.4592	0.5982	0.4760	0.5370
	H_E	0.5503	0.6044	0.5791	0.6095	0.5545	0.6747	0.5655	0.6454	0.5536	0.5882
	N_E	2.1855	2.4627	2.3346	2.5068	2.2052	2.7128	2.2555	2.9518	2.3151	2.5661
HMS6	PIC	0.7624	0.7952	0.6408	0.8124	0.6985	0.4853	0.4908	0.7680	0.5311	0.7779
	H_E	0.8042	0.8345	0.8740	0.8474	0.8915	0.9167	0.7665	0.8905	0.7614	0.8323
	N_E	4.8167	5.5728	3.2523	6.0845	3.8457	2.3607	2.3835	4.9622	2.5589	5.1636
HTG7	PIC	0.8684	0.8409	0.8511	0.8432	0.8689	0.8884	0.7275	0.8642	0.7231	0.8084
	H_E	0.8928	0.8673	0.8740	0.8689	0.8915	0.9167	0.7665	0.8905	0.7614	0.8323
	N_E	8.3333	6.8156	7.2746	6.9865	8.2492	9.7627	4.0902	8.0756	4.0223	5.8086
Mean PIC		0.7875	0.7749	0.7671	0.7741	0.7520	0.7401	0.7218	0.7923	0.7353	0.7967
Mean H_E		0.8259	0.8165	0.8075	0.8143	0.7985	0.7931	0.7730	0.8351	0.7823	0.8258
Mean N_E		6.7191	5.6989	6.0761	5.7059	5.8941	5.5498	5.4384	6.3297	5.7915	6.7076

PIC = Polymorphic information content; H_E = mean expected heterozygosity; N_E = mean effective number of alleles. DZ = Dezhou donkey; GY = Xiji donkey; GZ = Guanzhong donkey; GS = Gunsha donkey; JM = Jiami donkey; BY = Biyang donkey; QY = Qingyang donkey; TH = Taihang donkey; XJ = Xinjiang donkey; MG = Mongolia donkey.

Observed number of alleles (N_A) and allele frequencies for each donkey breed were also obtained. A total of 80 alleles were observed for the seven polymorphic loci in 415 individuals. Different loci had significantly different N_A. For instance, the maximum N_A was 15, which was observed at the AHT4 locus. In contrast, only six alleles were observed for the HMS7 locus. A total of 29 common alleles were found within the polymorphic loci, which were present in all ten breeds (Table 4).

Table 4. Common alleles of seven microsatellite markers in 10 domestic donkey breeds.

	AHT4	HTG6	HTG10	HMS3	HMS7	HMS6	HTG7
Common allele (bp)	155	87	104	161	108	165	138
	157	89	106	165	119	167	146
	159	91	114	167	121	175	150
		93	116	169		177	152
		97					

H_T, H_S, and G_{ST} are shown in Table 5. The G_{ST}, H_T and H_S for the seven polymorphic loci were 0.0154-0.0496, 0.5975-0.8965, and 0.5883-0.8707, respectively. The mean G_{ST} of seven loci was estimated to be 0.0341, which indicated that there was high genetic variability among these 10 donkey breeds.

Table 5. G_{ST}, H_T and H_S of seven microsatellite markers in 10 domestic donkey breeds.

Loci	G_{ST}	H_T	H_S
AHT4	0.0287	0.8965	0.8707
HTG6	0.0310	0.8600	0.8334
HTG10	0.0304	0.8899	0.8628
HMS3	0.0390	0.8799	0.8456
HMS7	0.0154	0.5975	0.5883
HMS6	0.0496	0.7630	0.7252
HTG7	0.0406	0.8797	0.8440
Total loci	0.0341	0.8238	0.7957

$G_{ST} = 1 - H_S/H_T$ (estimator of the degree of genetic differentiation); H_T = total population heterozygosity; H_S = subpopulation heterozygosity.

Genetic relationship among ten donkey breeds

Genetic distance can reflect the genetic variation and differentiation for different breeds. D_A and D_S between donkey breeds are shown in Table 6. These revealed that the genetic distance between Guanzhong donkey (GZ) and Biyang donkey (BY) (0.0179) and Qingyang donkey (QY) and XJ (0.0013) were smaller than 0.02, which indicated that GZ and BY, QY, and XJ had close genetic relationships. However, the furthest genetic distance was found between QY and DZ (0.2004) indicating that they had a more distant genetic relationship.

Table 6. Nei's genetic distance (D_A) and standard genetic distances (D_S) among 10 domestic donkey breeds.

Breeds	DZ	GY	GZ	GS	JM	BY	QY	TH	XJ	MG
DZ		0.1812	0.0675	0.1641	0.1091	0.1031	0.1806	0.1062	0.1587	0.1064
GY	0.1778		0.0492	0.0246	0.1113	0.1085	0.0767	0.1396	0.0859	0.1067
GZ	0.1021	0.0734		0.0725	0.0493	0.0179	0.0375	0.0967	0.0270	0.1299
GS	0.1621	0.1002	0.1025		0.0968	0.0988	0.1127	0.1717	0.0903	0.1145
JM	0.1312	0.0966	0.0641	0.1069		0.1397	0.1125	0.2080	0.0909	0.1492
BY	0.1373	0.1466	0.0894	0.1325	0.1425		0.0536	0.1349	0.0577	0.1336
QY	0.2004	0.1180	0.1033	0.1603	0.1437	0.1209		0.1637	0.0013	0.1269
TH	0.1439	0.1405	0.1236	0.1715	0.1491	0.1693	0.1591		0.1720	0.1611
XJ	0.1679	0.1118	0.0836	0.1171	0.1140	0.1202	0.0609	0.1589		0.1428
MG	0.1068	0.1222	0.1102	0.1208	0.1173	0.1144	0.1209	0.1334	0.1078	

Data below the diagonal represent D_A, while D_S are above the diagonal. DZ = Dezhou donkey; GY = Xiji donkey; GZ = Guanzhong donkey; GS = Gunsha donkey; JM = Jiami donkey; BY = Biyang donkey; QY = Qingyang donkey; TH = Taihang donkey; XJ = Xinjiang donkey; MG = Mongolia donkey.

The neighbor-joining tree based on D_A showed that all 10 donkey breeds could be clustered into two groups (Figure 1). Furthermore, the first group could be divided into two branches. The first branch included the Xiji (GY) and Gunsha donkeys, which clustered with JM and GZ donkeys. In the second branch, QY and XJ donkeys clustered first, followed by the BY donkey onto the same branch. In contrast, the DZ, Mongolia and Taihang donkeys were clustered in the second group.

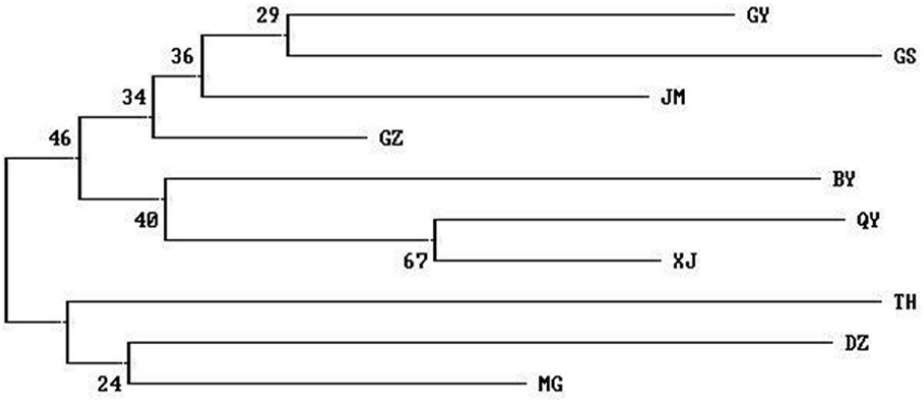

Figure 1. Neighbor-joining tree of ten donkey breeds based on D_A distances. GY = Xiji donkey; GS = Gunsha donkey; JM = Jiami donkey; GZ = Guanzhong donkey; BY = Biyang donkey; QY = Qingyang donkey; XJ = Xinjiang donkey; TH = Taihang donkey; DZ = Dezhou donkey; MG = Mongolia donkey.

DISCUSSION

In this study, we investigated polymorphisms at 10 microsatellite loci in 415 Chinese donkeys from 10 different breeds. Of those microsatellite loci, seven (HTG7, HTG10, AHT4, HTG6, HMS6, HMS3, and HMS7) were found to be highly polymorphic (PIC > 0.5), while three (HMS1, HTG4, and HMS5) were monomorphic. These results indicate that the Chinese domestic donkey possesses rich genetic diversity, and that the seven polymorphic microsatellite loci identified in this study are effective markers to analyze the genetic diversity and phylogenetic relationships between different donkey breeds.

Among the 10 microsatellites investigated in this study, the PIC of HTG4 and HMS5 were found to be 0.40 and 0.53 in five endangered Spanish donkey breeds, respectively, while both were monomorphic in Chinese donkey breeds (Aranguren-Méndez et al., 2002). In contrast, the locus HMS1 was found to be monomorphic in both Chinese and Spanish donkeys (Aranguren-Méndez et al., 2002). Generally, the Chinese donkey shows relatively higher genetic diversity than the Spanish donkey at the seven polymorphic loci investigated in the current study. In addition, the Chinese donkey population (0.8072) showed a relatively higher H_E than donkey populations (0.64) in the Croatian coastal region (Ivankovic et al., 2002). A previous study investigated polymorphisms of 24 microsatellite loci in eight Chinese donkey breeds representing large and medium-sized Chinese donkeys (Zhu and Su, 2011), and revealed a mean PIC of 0.6940, which is relatively lower than that found for the donkey populations in

the present study. Based on these results, we can conclude that the Chinese donkey possesses higher genetic diversity than donkeys from Spain and the Croatian coastal region.

The donkey is thought to have been imported into the Xinjiang region in China, from where it spread to the other regions of China via the Silk Road (Lei et al., 2007). Based on the neighbor-joining tree constructed using D_A, we found that there was a small genetic difference between XJ, QY, GY, and GZ donkeys, which indicates that they are closely related. These results can be explained by the fact that these donkey breeds are raised in Northwestern China where they are found in relatively close geographical locations. Therefore, the genetic relationships between donkey breeds are consistent with their geographical distribution.

The current study aimed to provide insight into the genetic relationships and diversity between Chinese donkey breeds, which will offer a valuable reference for rational strategies in donkey conservation and breeding programs.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Aranguren-Méndez J, Gómez M and Jordana J (2002). Hierarchical analysis of genetic structure in Spanish donkey breeds using microsatellite markers. *Heredity (Edinb)* 89: 207-211. http://dx.doi.org/10.1038/sj.hdy.6800117

Botstein D, White RL, Skolnick M and Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32: 314-331.

Green MR and Sambrook J (2012). Molecular cloning: A laboratory manual. 4th edn. Cold Spring Harbor Laboratory Press, New York.

Hou W and Hou B (2002). The breeding and meat of donkey. Golden Shield Press, Beijing.

Ivankovic A, Kavar T, Caput P, Mioc B, et al. (2002). Genetic diversity of three donkey populations in the Croatian coastal region. *Anim. Genet.* 33: 169-177. http://dx.doi.org/10.1046/j.1365-2052.2002.00879.x

Jordana J, Folch P and Aranguren J (2001). Microsatellite analysis of genetic diversity in the Catalonian donkey breed. *J. Anim. Breed. Genet.* 118: 57-63. http://dx.doi.org/10.1046/j.1439-0388.2001.00266.x

Lei CZ, Ge QL, Zhang HC, Liu RY, et al. (2007). African maternal origin and genetic diversity of Chinese domestic donkeys. *Asian-Aust. J. Anim. Sci.* 20: 645-652.

Ma YH, Xu GF, Wang DY and Liu HL (2002). Study on dynamic information of animal genetic resources in China. *Sci. Agric. Sinica.* 35: 552-555.

Nei M (1972). Genetic distance between populations. *Am. Nat.* 106: 283-292. http://dx.doi.org/10.1086/282771

Ota T (1993). Dispan: Genetic distance and phylogenetic analysis. Dissertation for PhD, Pennsylvania State University (USA).

Xie F (2004). Genetic variation within and relationships among populations of Chinese donkey breeds. Masters Dissertation. Yangzhou University (China).