Genetic Diversity of Chinese Indigenous Pig Breeds in Shandong Province Using Microsatellite Markers*

J. Y. Wang[1,2], J. F. Guo[1], Q. Zhang[2], H. M. Hu[1], H. C. Lin[1], Cheng Wang[1], Yin Zhang[1] and Y. Wu[1, **]

[1] Shandong Provincial Key Laboratory of Animal Disease Control and Breeding,
Institute of Animal Science and Veterinary Medicine,
Shandong Academy of Agricultural Sciences, Jinan 250100, China

ABSTRACT : To investigate the genetic diversity of six Chinese indigenous pig breeds in Shandong province (Laiwu Black, Dapulian Black, Licha Black, Yantai Black, Yimeng Black and Wulian Black), explain their genetic relationship and assess their integrity and degree of admixture with three Western commercial breeds (Landrace, Yorkshire and Duroc), 303 individuals from these breeds were genotyped for 26 microsatellite markers. In general, high genetic diversity (observed heterozygosity ranging from 0.5495 to 0.7746) and large breed differentiation (F_{ST} = 0.188) were observed. The indigenous pig breeds in Shandong exhibited consistently higher levels of genetic diversity than the three Western breeds. However, compared with the Western breeds, which have an F_{ST} value of 0.252, the indigenous breeds in Shandong have smaller F_{ST} value of 0.145. The analysis of breed relationship indicated that the six indigenous breeds are classified into two groups. One includes four breeds, Licha, Yantai, Yimeng and Wulian, which have experienced large gene introgression of the Western breeds through progressive crossbreeding as well as gene flow among themselves. The other includes Laiwu and Dapulian, which are less influenced by the Western breeds and other indigenous breeds in Shandong in the recent past. The results show that some measures must be taken to effectively protect these indigenous pig breeds in Shandong. (**Key Words :** Microsatellite Marker, Genetic Diversity, Chinese Indigenous Pig Breeds, Shandong Province)

INTRODUCTION

China is one of the countries possessing the most abundant genetic resources of pig breeds in the world. One hundred and twenty-eight Chinese indigenous pig breeds are documented in the Domestic Animal Diversity Information System (DAD-IS) of the Food and Agriculture Organization (FAO) (http://dad.fao.org/), which constitutes almost one-third of all pig breeds in the world. Shandong province, with an area of more than 150,000 km^2 and a diverse terrain, is one of the major districts of pig production in China. Historically, many indigenous pig breeds were developed in Shandong and all the presently existing breeds include Laiwu Black, Dapulian Black, Licha Black, Yantai Black, Yimeng Black and Wulian Black (Si et al., 1999). All of these breeds have their special characteristics, such as high prolificacy, good meat quality and strong disease-resistance, but their growth and carcass performances are much lower than that of the commercial breeds from Europe and North America. Among these breeds, Laiwu, Dapulian, Licha, and Yimeng are documented in DAD-IS. The other two, Wulian and Yantai, although not in DAD-IS, have their long histories and unique characteristics, too.

Yorkshire was firstly introduced into Shandong and raised in the area of Qingdao during the period of Guangxu of Qing Dynasty (Zhang, 1986). From then on, a small scale of crossbreeding between indigenous breeds and Western breeds had been proceeding. From the eighties of the 20th

century, however, larger scale of importation of Western commercial pig breeds into Shandong has been proceeding for commercial interest. As the result, the indigenous pig breeds in Shandong, as well as many other indigenous breeds in China have been in direct competition with the highly selected Western breeds, and the population sizes of many of them decrease dramatically and are in danger of being replaced, or hybridized with Western commercial breeds (Fang et al., 2005). In recent years, increased awareness of the value and importance of local genetic resource, as well as the consumer demands for high-quality meat products, have turned attention back to the traditional breeds and efforts have been made toward recovering those nearly extinct genetic resources. Although in Shandong six indigenous pig breeds are still existing now (Si et al., 1999), most of them have been crossed with the Western commercial breeds. However, the pattern and extent of the admixture remains unclear.

So far, the genetic diversity of some Chinese indigenous pig breeds has been evaluated by microsatellite markers (Fan et al., 2002; Yang et al., 2003b; Zhang et al., 2003; Li et al., 2004; Fang et al., 2005; Megens et al., 2008; Chang et al., 2009) and mitochondrial DNA (mtDNA) sequence (Yang et al., 2003a; Fang and Andersson, 2006; Wu et al., 2007; Wang et al., 2009 and 2010). However, the vast genetic resources in China are still poorly characterized except for a few very famous breeds. Of the six pig breeds in Shandong, only Laiwu (Zhang et al., 2003), Yimeng (Zhang et al., 2003; Fang et al., 2005) and Licha (Megens et al., 2008) were included in the previous studies using microsatellite markers. Therefore, a comprehensive assessment of their genetic diversity and relationship with the Western breeds represents a major step toward the development of conservation and improvement programs.

In the present study, we used 26 microsatellite markers, recommended by the Food and Agriculture Organization (FAO) of the United Nations and the International Society of Animal Genetics (ISAG), to elucidate the degree and pattern of genetic variability in the six indigenous pig breeds in Shandong, explain their genetic relationship, and assess their integrity and degree of admixture with the Western commercial breeds, Landrace, Yorkshire and Duroc, mainly used in Shandong.

MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 303 individuals representing the nine pig breeds were used in this study (Table 2). Ear samples for the indigenous breeds were collected from their conservation farms (detailed information of the geographic regions of sampling is shown in Figure 1). Under the pressure of market competition and financial constraint, the sizes of

Figure 1. The geographical distribution of six indigenous pig breeds of Shandong province and three Western commercial pig breeds. LW = Laiwu Black; DP = Dapulian Black; LC = Licha Black; YM = Yimeng Black; YT = Yantai Black; WL = Wulian Black; D = Duroc; Y = Yorkshire; C = Landrace. The names and location of the capital city (Jinan) and other main cities of 16 districts of Shandong province are given in the map too.

above conservation populations were rather small, resulting in a relative small proportion of male individuals for these breeds. Individuals sampled of the five breeds, laiwu, Dapulian, Licha, Yantai and Yimeng were unrelated (without common grandparents). The breed, Wulian, does not have pedigree records, so the samples were randomly collected. Ear samples for the three Western commercial breeds were collected from the Research Center of Pig Breeding and Reproduction in Shandong province. All individuals within these breeds were unrelated based in the pedigree information. Total genomic DNA was extracted from ear tissues by the standard phenol-chloroform method (Sambrook and Russell, 2001).

Microsatellite genotyping

A panel of 26 microsatellite markers, randomly located on 16 chromosomes, as recommended by FAO-ISAG (http://www.toulouse.inra.fr/lgc/pig/panel/panel2004.htm), was selected for genotyping. One of each paired primer was 5' - end labeled with fluorescence dye (FAM or HEX). Microsatellite markers were amplified by PCRs in 10-μl final volumes with 0.5 μl of 40 ng/μl DNA, 1 μl of 10× buffer, 0.8 μl of 10 mmol/L dNTP, 0.5 μl of each primers (12.5 μmol/L forward and reverse primers), and 0.5U of Taq Hs DNA polymerase (TaKaRa, Japan). The thermal cycling conditions in PTC-2000 DNA Engine (Bio-Rad, USA) included an initial denaturation for 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature (ranging from 50°C to 60°C), 30 s at 72°C, and a final extension step of 72°C for 20 min. For genotyping of samples, PCR products of up to 3 or 4 markers were combined and analyzed using an ABI 377 DNA sequencer

(Applied Biosystems, USA). The fluorescent-labeled marker HD-400 (Applied Biosystems) was used as an internal size standard for length calibration. Fragment length of the PCR products was determined using GeneScan software (Version 3.0, Applied Biosystems).

Data analyses

Total number of alleles (TNA) per locus, the mean number of allele (MNA) per locus, observed heterozygosity (H_O), and expected heterozygosity (H_E) under the Hardy-Weinberg assumption for each locus were computed using the Excel Microsatellite Toolkit 3.1 software (Park, 2001). To compare the number of alleles between different sample sizes, allelic richness (A_R) which measures the number of allele independent of sample sizes, was calculated using the program FSTAT v.2.9.3 software package (Goudet, 2001). Wright's F-statistics (F_{ST}, F_{IS} and F_{IT}) for each marker were also calculated using the program FSTAT as well by Weir and Cockerman's method (1984). The probability test approach described by Guo and Tomson (1992) and implemented in the GENEPOP 4.0 software (Rousset, 2008) was employed to test for Hardy-Weinberg equilibrium. The length of the Markov chain was set to 10000 iterations per batch for 1,000 batches and the dememorization number was 10,000. The genetic diversity between the populations based on allele frequencies was calculated according to Nei's standard genetic distance (Nei, 1972) using the DISPAN program (Ota, 1993). Phylogenetic trees were constructed using the neighbour-joining (NJ) clustering (Saitou and Nei, 1987) and the unweighted pair group method with the arithmetic mean (UPGMA; Sneath and Sokal, 1973) from Nei's standard genetic distance using DISPAN too. Bootstrap (Felsenstein, 1985) re-sampling (n = 1,000) was performed to test the robustness of the tree topologies. Based on the genotypes at the 26 microsatellite markers, individual animals were clustered into a given number of populations and assigned probabilistically to clusters inferred with a Bayesian approach implemented by the STRUCTURE software (Pritchard et al., 2000). The tests were done based on an admixture model where the allelic frequencies were correlated applying burn-in period of 10,000 and 100,000 iterations for data collection. Two to twelve inferred clusters were performed with three independent runs each.

RESULTS

Microsatellite markers

A total of 269 alleles (Table 1) were observed at the 26 markers distributed on 16 chromosomes. TNA per locus was 10.35, ranging from 7 (S0090, S0143, Sw72) to 18 (Sw1067). The observed amplitude of more than half markers in allele sizes exceeded the expected range,

indicating that some new alleles were present in the populations analyzed. The H_O ranged from 0.5505 to 0.8075, with a mean value of 0.6868, which was larger than H_E (mean value 0.6565). Overall, private alleles (i.e., found exclusively in one breed) were detected in 19 markers and in all breeds except Laiwu. Wulian had the largest number of private alleles which distributed in nearly half of the markers analyzed, while Dapulian had the second largest, which distributed in nearly one-third markers. Significant deviations (p<0.05) from Hardy-Weinberg equilibrium were observed for all 26 markers. On average across markers, about 28 percent of the markers did not comply with the Hardy-Weinberg equilibrium (p<0.05) in the nine breeds studied.

Intrapopulation genetic variation

The within-breed analyses (Table 2) showed that all of the six indigenous pig breeds had larger genetic diversity than the three Western commercial breeds in all measures of genetic diversity (MNA, A_R, H_O and H_E). Among the six indigenous breeds in Shandong, Wulian had the largest level of genetic diversity (H_O = 0.7746), while Laiwu had the smallest level genetic diversity (H_O = 0.6681). Among the three Western commercial breeds, Duroc had the smallest genetic diversity with H_O of 0.5495, while Yorkshire had the largest one, with the H_O of 0.6311.

Interpopulation genetic variation

The breed differentiation measured by Wright's F-statistics (F_{IT}, F_{IS}, and F_{ST}) is shown in Table 1 and 2. The divergence between expected and observed heterozygosity measured by the F_{IT} statistic, had a global mean of 0.147 for all markers, and ranged between 0.045 and 0.248. The within-breed excess in heterozygosity measured by the F_{IS} statistic had a global mean of -0.051, and ranged between -0.168 to 0.039. The genetic differentiation among breeds measured by the F_{ST} statistic had a global mean of 0.188, and ranged between 0.127 and 0.283, indicating that 81.2% of the genetic variability was caused by the differences among individuals within breeds and 18.8% was due to the differentiation among breeds. Compared between the two groups, the one of indigenous breeds had a rather smaller F_{ST} of 0.145 than that (0.252) of the one of Western commercial breeds.

Interpopulation relationship

Different genetic distance measures were estimated but all showed a very high correlation so that only Nei's standard genetic distances were reported here (Table 3). The genetic distances between the three Western breeds ranged from 0.4905 to 0.6662. They had larger genetic distances with Laiwu and Dapulian, ranging from 0.7581 to 1.2122, than with the other four breeds, Licha, Yantai, Yimeng and

Table 1. Microsatellite markers, allele range, total number of alleles (TNA) per locus, breeds with private alleles detected, expected heterozygosity (H_E), observed heterozygosity (H_O), the Wright's F-statistics (F_{IS}, F_{IT}, F_{ST}), and proportions of breeds not complying with the Hardy-Weinberg equilibrium (PBHW) at $p<0.05$ for each marker in nine pig breeds studied

Markers	Allele range (bp)	TNA	Breeds with private alleles	H_E	H_O	F_{IT}	F_{ST}	F_{IS}	PBHW
Sw2410	90-110	9	Dapulian	0.6386	0.6235	0.214	0.193	0.025	0.33
Sw830	144-164	9	Yorkshire, Dapulian	0.5680	0.6160	0.060	0.141	-0.094	0.11
Sw911	107-137	9	Wulian	0.6231	0.6202	0.199	0.194	0.006	0.11
Sw240	178-216	15	Yimeng, Wulian	0.7108	0.7615	0.125	0.181	-0.069	0.22
S0068	244-274	16	Landrace	0.7849	0.8075	0.102	0.127	-0.029	0.33
Sw1067	95-135	18	Dapulian, Yimeng, Wulian	0.7236	0.7651	0.164	0.210	-0.058	0.33
Sw2008	148-178	8	Wulian	0.5735	0.6084	0.135	0.188	-0.065	0.44
S0101	202-224	10	Dapulian, Yantai	0.6393	0.6911	0.131	0.197	-0.083	0.22
Swr1941	94-114	11	Yorkshire	0.6459	0.6625	0.181	0.204	-0.028	0.33
Sw1828	220-248	8		0.6837	0.7099	0.135	0.165	-0.036	0.44
Sw632	110-130	9	Licha	0.7014	0.7095	0.181	0.192	-0.017	0.22
S0090	137-159	7	Wulian	0.6589	0.7101	0.075	0.145	-0.082	0.33
S0178	92-124	11		0.6616	0.6377	0.239	0.208	0.039	0.22
IGF1	193-209	9	Wulian	0.7143	0.7828	0.052	0.140	-0.099	0.22
S0143	222-256	7	Wulian	0.5723	0.6601	0.045	0.180	-0.165	0.44
Sw122	100-116	9	Wulian	0.6893	0.7136	0.166	0.199	-0.041	0.33
Sw72	227-251	7		0.6852	0.7283	0.117	0.176	-0.072	0.11
Sw857	89-103	10	Yorkshire, Dapulian	0.6856	0.7157	0.156	0.192	-0.044	0.11
S0155	136-178	8		0.6277	0.6375	0.239	0.252	-0.018	0.22
Sw936	197-225	8		0.5832	0.6132	0.197	0.240	-0.058	0.22
Sw2406	84-112	12		0.6019	0.5938	0.230	0.227	0.004	0.22
S0002	151-167	17	Duroc, Licha, Wulian	0.7471	0.7654	0.116	0.138	-0.025	0.33
S0026	210-260	10	Licha, Wulian	0.6488	0.7492	0.053	0.190	-0.168	0.44
S0355	101-131	10		0.5275	0.5505	0.248	0.283	-0.049	0.22
Sw24	150-176	12	Dapulian	0.7166	0.7344	0.140	0.168	-0.033	0.44
S0228	184-242	10	Dapulian, Wulian	0.6566	0.6893	0.118	0.167	-0.059	0.33
Average		10.35		0.6565	0.6868	0.147	0.188	-0.051	0.28

Table 2. Groups, breeds, the population size of conservation farms, number of samples analyzed (No.), mean number of alleles per locus (MNA), mean allelic richness (A_R), expected heterozygosity (H_E), observed heterozygosity (H_O), proportion of markers not in the Hardy-Weinberg equilibrium (PLHW) at $p<0.05$, the Wright's F-statistics (F_{ST}) for each breed

Groups	Breeds	Population size	No.	MNA	A_R	PIC	H_O	H_E	PLHW	F_{ST}
Indigenous pig breeds in Shandong	Laiwu	139	30	5.15	5.00	0.5606	0.6681	0.6211	0.3462	-
	Dapulian	79	36	6.00	5.65	0.6582	0.7678	0.7159	0.5000	-
	Yimeng	84	36	5.23	4.97	0.5994	0.7137	0.6571	0.0769	-
	Yantai	54	36	5.35	5.07	0.6269	0.7553	0.6825	0.3846	-
	Licha	101	25	5.69	5.67	0.6578	0.7115	0.7156	0.1923	-
	Wulian	40	32	8.04	7.77	0.7645	0.7746	0.8048	0.2692	-
	Mean	82.83	32.5	5.91	5.6883	0.6446	0.7318	0.6995	0.2949	0.145
Western commercial pig breeds	Landrace	69	36	4.08	3.90	0.5387	0.6311	0.5991	0.1923	-
	Yorkshire	111	36	4.46	4.30	0.5184	0.6096	0.5813	0.3462	-
	Duroc	98	36	3.85	3.69	0.4671	0.5495	0.5312	0.2308	-
	Mean	92.67	36	4.13	3.9633	0.5081	0.5967	0.5705	0.2564	0.252
Means of all breeds analyzed		86.11	33.67	5.32	5.11	0.5991	0.6868	0.6565	0.2821	0.188

Table 3. Nei's standard genetic distances between nine pig breeds studied based on 26 microsatellite markers
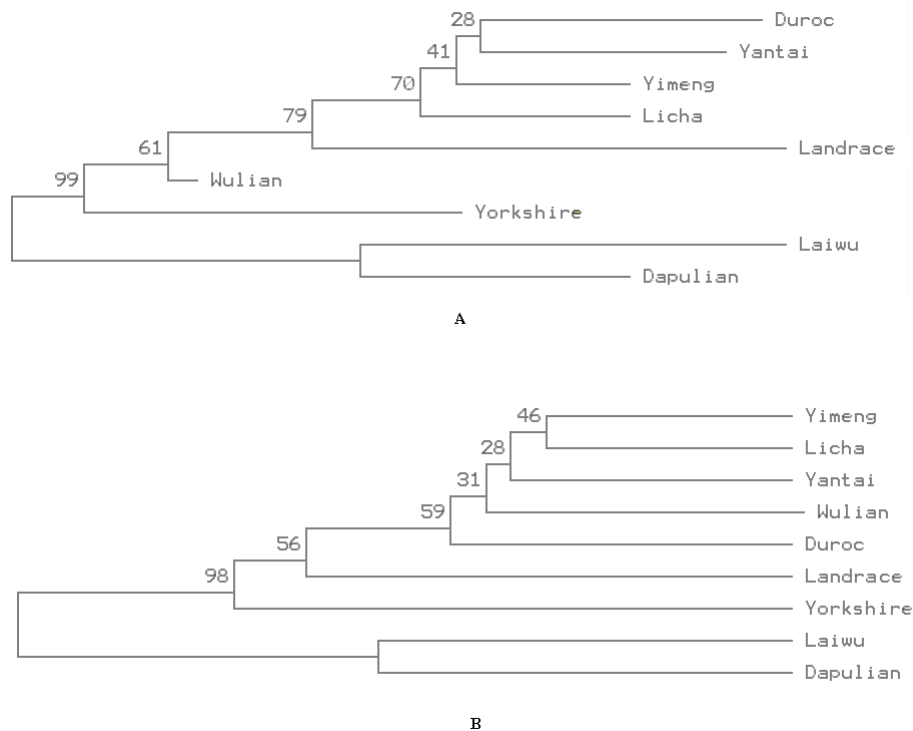
	Yorkshire	Landrace	Duroc	Laiwu	Dapulian	Yimeng	Yantai	Licha	Wulian
Yorkshire									
Landrace	0.6195								
Duroc	0.6662	0.4905							
Laiwu	0.7581	1.0551	1.2122						
Dapulian	0.8387	0.9094	0.9541	0.4190					
Yimeng	0.5063	0.4892	0.2872	0.8895	0.7088				
Yantai	0.5991	0.5981	0.3208	0.6616	0.6956	0.2689			
Licha	0.6050	0.4916	0.3430	0.8322	0.6570	0.2450	0.3034		
Wulian	0.3434	0.3751	0.4194	0.4877	0.4820	0.3276	0.3275	0.2801	

Wulian, ranging from 0.2872 to 0.6050. Among the six indigenous breeds in Shandong, Licha, Yantai, Yimeng and Wulian, had relative small genetic distances with each other, ranging from 0.2450 to 0.3276, while the genetic distance between Laiwu and Dapulian was a little larger (0.4190). On the other hand, the four breeds, Licha, Yantai, Yimeng and Wulian, had large genetic distances with Laiwu and Dapulian, ranging from 0.4820 to 0.8895, even larger than them with the three Western breeds.

To understand the relationship among the nine pig breeds, NJ and UPGMA phylogenetic trees (Figure 2) were constructed based on Nei's standard genetic distances. The tree topologies were confirmed by relatively high bootstrap values. Trees from both NJ and UPGMA methods showed similar topologies, but both trees did not clearly divide the indigenous breeds in Shandong and the Western commercial breeds into different clusters. Four indigenous pig breeds in Shandong, Yimeng, Yantai, Licha and Wulian, and the three Western pig breeds grouped together into cluster I, while the other two indigenous breeds, Laiwu and Dapulian, made up of the cluster II. The difference of the two trees was that, in NJ, Duroc grouped together firstly with Yantai, then with Yimeng, Licha, Landrace, Wulian, and finally with Yorkshire, while in UPGMA, four indigenous pig breeds in Shandong grouped together firstly, then with Duroc, Landrace, and finally with Yorkshire.

To supplement F_{ST} analyses, genetic structure analysis using a Bayesian approach by STRUCTURE software
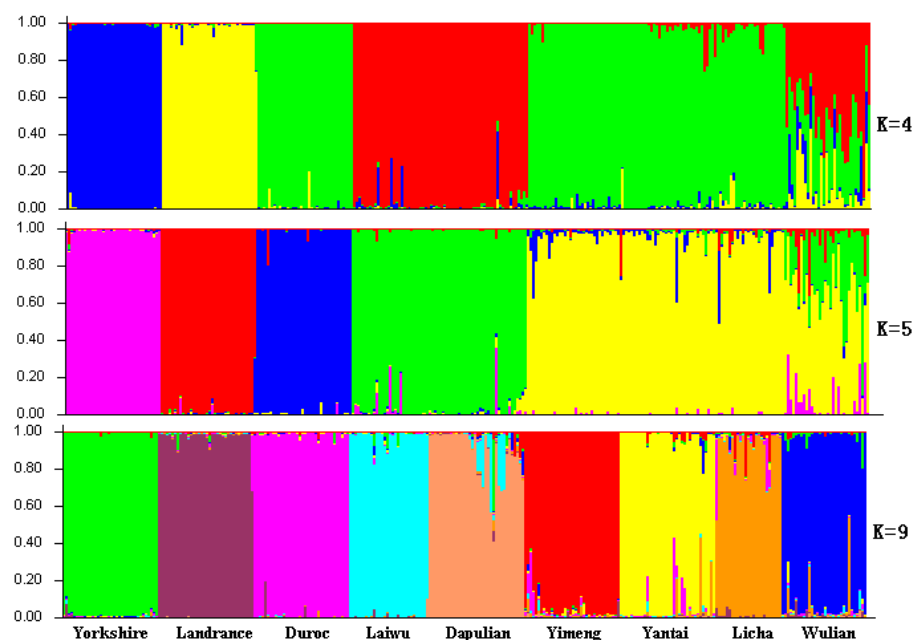

A

B

Figure 2. Phylogenetic trees of the six indigenous pig breeds in Shandong province and the three Western commercial pig breeds based on Nei's standard genetic distances. The values at the nodes are the percentages of bootstrap values from 1,000 replications of re-samplings. A: Neighboring-joining (NJ) tree; B: Unweighted pair group method with the arithmetic mean (UPGMA) tree.

Table 4. Proportional contribution of the inferred cluster (K = 9) to the breeds studied

Breeds	Clusters								
	1	2	3	4	5	6	7	8	9
Yorkshire	0.980	0.004	0.003	0.002	0.001	0.004	0.002	0.002	0.002
Landrace	0.006	0.970	0.012	0.002	0.002	0.003	0.002	0.002	0.002
Duroc	0.003	0.009	0.971	0.001	0.001	0.005	0.004	0.003	0.002
Laiwu	0.026	0.004	0.003	0.946	0.005	0.003	0.005	0.003	0.005
Dapulian	0.015	0.004	0.003	0.060	0.875	0.023	0.003	0.007	0.010
Yimeng	0.004	0.006	0.023	0.004	0.003	0.941	0.008	0.007	0.005
Yantai	0.006	0.003	0.031	0.006	0.003	0.013	0.899	0.030	0.009
Licha	0.004	0.010	0.041	0.003	0.002	0.032	0.008	0.892	0.007
Wulian	0.018	0.009	0.006	0.010	0.006	0.007	0.009	0.034	0.901

(Pritchard et al., 2000) was performed with increasing numbers of inferred populations. Panels with K = 4 inferred clusters, Yorkshire (blue) and Landrace (Yellow), Duroc (green) and Laiwu and Dapulian (red) were discriminated (Figure 3). When K = 5, the four indigenous pig breeds in Shandong, Licha, Yantai, Yimeng and Wulian, were further separated (Yellow) from Yorkshire (pink) and Landrace (red), Duroc (blue) and Laiwu and Dapulian (green). Progressively, as K increased, the contributions of the assumed populations resulted in the complete separation of the nine breeds, which were essentially identified with each one of the ancestral populations. When the number of clusters assumed was the same as the number of breeds, the proportional contribution of the assumed ancestral populations to each one of the current breeds was computed, and the corresponding results were summarized in Table 4. Each one of the breeds was very closely identified with one of the "ancestral" populations, from which it received a contribution to its gene pool of at least 0.875 (Dapulian). Although only Laiwu had unnegligible contribution (larger than 0.05) to Dapulian, there were large differences of ancestral contribution among individuals within breed in the indigenous pig breeds in Shandong, as shown by the diagram (Figure 3).

Figure 3. Clustering assignment of the six indigenous pig breeds in Shandong province and the three Western commercial pig breeds obtained by STRUCTURE analyses. Each of the 303 animals is represented by a thin vertical line that is divided into segments whose size and color correspond to the relative proportion of the animal genome corresponding to a particular cluster. Panels with K = 4 inferred clusters, Yorkshire (blue) and Landrace (Yellow), Duroc (green) and Laiwu and Dapulian (red) are discriminated; with K = 5, four indigenous pig breeds in Shandong, Yimeng, Yantai, Licha and Wulian are further separated (Yellow) from Yorkshire (pink) and Landrace (red), Duroc (blue) and Laiwu and Dapulian (green); with K = 9 inferred clusters corresponding to the nine breeds, complex breed admixture patterns can be visualized.

DISCUSSION

In our study, the set of microsatellite markers recommended by FAO-ISAG was used for analyzing the genetic diversity of six Chinese indigenous pig breeds in Shandong province and the relationship among them and the three Western commercial breeds. The indigenous pig breeds in Shandong exhibit consistently higher genetic diversity, with H_O ranging from 0.6681 to 0.7746, than the three Western commercial pig breeds, with the H_O ranging from 0.5495 to 0.6311. There results are in general congruent with the previous studies (*e.g.,* Zhang et al., 2003; Fang et al., 2005; Megens et al., 2008) that Chinese indigenous pig breeds have larger genetic diversity than Western commercial breeds. Moreover, compared with other Chinese indigenous breeds, indigenous breeds in Shandong belong to those which exhibit high leveler of genetic variation among the Chinese indigenous breeds (*e.g.,* Zhang et al., 2003; Fang et al., 2005; Megens et al., 2008).

Just like other indigenous pig breeds in China, the indigenous pig breeds in Shandong mainly have been raised in the extensive production system. Unlike the Western commercial pig breeds, they have not gone through the high selection pressure. It is the main reason for their high genetic diversity. Of the six indigenous breeds in Shandong, Wulian has the highest genetic diversity (H_O = 0.7746) while Laiwu has the lowest (H_O = 0.6681). Their genetic diversity levels are consistent with their conservation states, or the introgression of Western commercial pig breeds into the indigenous breeds, as shown in Figure 2 and 3.

For all loci combined, on average, about 28 percent of the breed-loci combinations did not comply with Hardy-Weinberg equilibrium (p<0.05), mostly because of a larger H_O than H_E in 21 loci. Several factors, such as non-random mating, Walhund effect, sampling error and the less proportion of male individuals, may cause the deviation from Hardy-Weinberg equilibrium and higher H_O than H_E in this study.

In both NJ and UPGMA phylogenetic trees (Figure 2), the six indigenous pig breeds in Shandong and the three Western commercial breeds are not clearly divided into different clusters. Clustering assignment (Figure 3) also suggests that a complex admixture between the indigenous breeds in Shandong and the Western breeds. These indicate that the indigenous breeds in Shandong, especially Licha, Yantai, Yimeng and Wulian, have experienced large gene introgression of Western breeds through progressive crossbreeding with these breeds. Three of the six breeds, Yimeng, Licha and Laiwu, had been used in other studies (Zhang et al., 2003; Fang et al., 2005; Megens et al., 2008). In these studies, all the indigenous breeds of Shandong have closer relationship with Western commercial breeds than other Chinese indigenous breeds and are placed in the peripheral of the cluster of the Chinese indigenous breeds.

Compared with the three Western commercial breeds, which have an F_{ST} of 0.252, the six indigenous breeds in Shandong have a rather smaller F_{ST} of 0.145. According to their different genetic pattern revealed by phylogenetic trees (Figure 2) and Clustering assignment (Figure 3), the six breeds can be classified into two groups. One includes four breeds, Licha, Yantai, Yimeng and Wulian, more influenced by Western commercial breeds. The four indigenous breeds mainly distribute in the East of Shandong, where the Western breeds were imported firstly and economy is relatively developed. According to the record (Si et al., 1999), Licha was influenced by Yorkshire in 1940s, Habai (hybrid by Berkshire and one indigenous breed of Northeast of China) in 1957, Landrance in 1970, Duroc in 1990s respectively. Yantai was influenced firstly by Berkshire in 1940s, then Landrance and Middle Yorkshire in 1950s, finally Duroc in 1990s respectively. Yimeng was influenced firstly by Berkshire in 1930s, later Middle Yorkshire, finally Duroc at the end of the 20 century. Wulian was influenced by Berkshire at about 1940. Besides it, no crossbreeding history had been recorded. However, until now the conservation farm of Wulian has not been completely built, and there must have been crossbreeding with Western breeds unrecorded as revealed by this study. The other includes Laiwu and Dapulian, less influenced by Western commercial breeds. The two breeds mainly distribute in the Middle of Shandong, where the economy and transportation are relatively undeveloped. According to the record (Si et al., 1999), Laiwu was influenced firstly by Middle Yorkshire and Xinjin in 1950s, then Neijing in 1970s. Dapulian was influenced firstly by Xinjin, Middle Yorkshire and Berkshire during 1950s to 1960s and then Neijing and Jihua in 1970s. Xijin, Neijing, Xihua and Jihua are breeds formed by hybridization of Western breeds with the indigenous breeds of other province of China. Conservation farms of Laiwu and Dapulian were built at the beginning of 1980s. Though complicate crossbreeding happened with other breeds during 1950s to 70s, Laiwu and Dapulian are conserved better and influenced less than the other four indigenous breeds by Western commercial breed.

Licha, Yantai, Yimeng and Wulian, have very close relationship, with genetic distance ranging from 0.2450 to 0.3276. Clustering assignment using STRUCTURE with K = 5 could not separate them from each other. Their close relationship consists in the following two reasons. Firstly, they are all influenced by Western breeds. Of the three Western breeds, Licha, Yantai and Yimeng, are influenced mostly by Duroc, while Wulian by Yorkshire. Although the ancestral contributions of Western breeds are all under 0.05 (Table 4), there are several individuals of each breed, for which the ancestral contributions of Western breeds are much higher than the average (data not shown), suggesting

in the current conservation farm, the crossbreeding with Western commercial breeds may be still going on in small scale. Secondly, there is frequent gene flow among them. There are also some individuals of each breed, for which the ancestral contributions of other breeds are much higher than the average (data not shown), indicating the gene flow happened in the recent generations and is not yet stabilized. As we know that in some conservation farms of them, phenotypically alike indigenous pigs of other breeds are sometimes introduced to reduce the inbreeding of their population.

Laiwu and Dapulian were grouped in a separate cluster in both NJ and UPGMA phylogenetic trees (Figure 2) with a close genetic distance of 0.4190. They have large genetic distances with the Western breeds (ranging from 0.7582 to 1.2122) as well as the other four indigenous breeds in Shandong (ranging from 0.4820 to 0.8895). From the clustering assignment (Figure 3) using STRUCTURE with K = 4, they were discriminated from Western commercial breeds and the other four indigenous breeds in Shandong. These results suggest that that these two breeds are less influenced by Western pig breeds except for some ancestral contribution of Yorkshire into them, and gene flow between them and the other indigenous breeds in Shandong is also rather weak. In our previous study using the control region of mtDNA (Wang et al., 2009 and 2010), these two breeds are also grouped into relatively respective sub-clusters in the NJ phylogenetic tree, suggesting there is less gene admixture between them and the other breeds in maternal lineage.

Dapulian, meaning a kind of flower in its Chinese name, has flowerlike wrinkles on the forehead. In addition, it has higher figure and larger body weight than Laiwu. Although the large difference in appearance exists between them, they have rather small genetic distance (0.4190). One reason for their close relationship is the admixture of Laiwu in Dapulian, as showed in Clustering assignment (Figure 3). The main distribution areas of Laiwu and Dapulian are contiguous. With the increment of communication and the reduction of number of Dapulian pigs, it is very possible that it is influenced by Laiwu, whose conservation farm was built much earlier. Additionally, the other reason is that it is common that Chinese indigenous pig breeds have close genetic distance though their great appearance difference, as reported by other studies (Fan et al., 2002; Yang et al., 2003a; Li et al., 2004; Fang et al., 2005), too.

So far, all of the six indigenous pig breeds in Shandong have their own conservation farms, built according to their geographic distribution and appearance. However, with limited financial support, the population sizes are very small in most conservation farms, with some verging on extinction. To reduce the conservation cost and increase

effective population size, it is advisable to cross pig breeds of the same sub-cluster based on their phylogenetic relationship (Fang et al., 2005; Megens et al., 2008). The four breeds of the first group of the indigenous breeds in Shandong, Licha, Yantai, Yimeng and Wulian, have close genetic distances with each other, and their characteristics in appearance and performance are also rather similar. Therefore, their conservation farms can be reorganized into one or two according to the practical considerations. As for Laiwu and Dapulian, due to their uniqueness, they should be protected well in order to maintain their future utilization. Our results also reveal noticeable ancestral contribution of Yorkshire exists in them and some Dapulian is obviously influenced by Laiwu. It is necessary to purify them by culling the individuals having other ancestral contribution through testing the replacement pigs using microsatellite markers.

REFERENCES

Chang, W. H., H. P. Chu, Y. N. Jiang, S. H. Li, Y. Wang, C. H. Chen, K. J. Chen, C. Y. Lin and Y. T. Ju. 2009. Genetic variation and phylogenetics of Lanyu and exotic pig breeds in Taiwan analyzed by nineteen microsatellite markers. J. Anim. Sci. 87(1):1-8.

Fan, B., Z. G. Wang, Y. J. Li, X. L. Zhao, B. Liu, S. H. Zhao, M. Yu, M. H. Li, S. L. Chen, T. A. Xiong and K. Li. 2002. Genetic variation analysis within and among Chinese indigenous swine populations using microsatellite markers. Anim. Genet. 33(6):422-427.

Fang, M. and L. Andersson. 2006. Mitochondrial diversity in European and Chinese pigs is consistent with population expansions that occurred prior to domestication. Proc. R. Soc. B. 273(1595):1803-1810.

Fang, M., X. Hu, T. Jiang, M. Braunschweig, L. Hu, Z. Du, J. Feng, Q. Zhang, C. Wu and N. Li. 2005. The phylogeny of Chinese indigenous pig breeds inferred from microsatellite markers. Anim. Genet. 36(1):7-13.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Goudet, J. 2001. Fstat, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.Unil.Ch/izea/softwares/fstat.Html.

Guo, S. W. and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48(2):361-372.

Jiang, Y. N., C. Y. Wu, C. Y. Huang, H. P. Chu, M. W. Ke, M. S. Kung, K. Y. Li, C. H. Wang, S. H. Li, Y. Wang and Y. T. Ju. 2008. Interpopulation and intrapopulation maternal lineage genetics of Lanyu pig (*Sus scrofa*) by analysis of mitochondrial cytochrome b and control region sequences. J. Anim. Sci. 86:2461-2470.

Li, S. J., S. L. Yang, S. H. Zhao, B. Fan, M. Yu, H. S. Wang, M. H. Li, B. Liu, T. A. Xiong and K. Li. 2004. Genetic diversity analyses of 10 indigenous Chinese pig populations based on 20 microsatellites. J. Anim. Sci. 82(2):368-374.

Megens, H. J., R. P. Crooijmans, M. San Cristobal, X. Hui, N. Li

and M. A. Groenen. 2008. Biodiversity of pig breeds from China and Europe estimated from pooled DNA samples: Differences in microsatellite variation between two areas of domestication. Genet. Sel. Evol. 40(1):103-128.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106:283-292.

Ota, T. 1993. Dispan: Genetic distance and phylogenetic analysis. Pennsylvania State University, USA.

Park, S. D. E. 2001. Trypanotolerance in West African cattle and the population genetic effects of selection. [ph.D.Thesis], University of Dublin.

Pritchard, J. K., M. Stephens and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol. Ecol. Resour. 8(1):103-106.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425.

Sambrook, J. and D. Russell. 2001. Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Si, J. C., H. B. Zhang and J. J. Tang. 1999. Livestock breeds in Shandong province. Haitian Press, Shenzhen, China.

Sneath, P. H. A. and R. R. Sokal. 1973. Numerical taxonomy. W. H. Freeman, San Francisco.

Wang, J. Y., J. F. Guo, S. L. Sun, C. Wang, Y. Zhang and Y. Wu. 2009. The genetic diversity and phylogenetic relationship among pig breeds of Shandong province based on partial sequence of mtDNA D-Loop region. Chinese J. Anim. Vet. Sci. 40(6):792-799.

Wang, J. Y., J. F. Guo, X. J. Hao, H. M. Hu, H. C. Lin, Y. Zhang, C. Wang and Y. Wu. 2010. Phylogenetic relationship of pig breeds from Shandong province of China and their influence by modern commercial breeds by analysis of mitochrondrial DNA sequences. Ital. J. Anim. Sci. 9:e48.

Weir, B. S. and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution. 38(6):1358-1370.

Wu, G. S., Y. G. Yao, K. X. Qu, Z. L. Ding, H. Li, M. G. Palanichamy, Z. Y. Duan, N. Li, Y. S. Chen and Y. P. Zhang. 2007. Population phylogenomic analysis of mitochondrial DNA in wild boars and domestic pigs revealed multiple domestication events in eastern Asia. Genome Biol. 8(11):R245.

Yang, J., J. Wang, J. Kijas, B. Liu, H. Han, M. Yu, H. Yang, S. Zhao and K. Li. 2003a. Genetic diversity present within the near-complete mtDNA genome of 17 breeds of indigenous Chinese pigs. J. Hered. 94(5):381-385.

Yang, S. L., Z. G. Wang, B. Liu, G. X. Zhang, S. H. Zhao, M. Yu, B. Fan, M. H. Li, T. A. Xiong and K. Li. 2003b. Genetic variation and relationships of eighteen Chinese indigenous pig breeds. Genet. Sel. Evol. 35(6):657-671.

Zhang, G. X., Z. G. Wang, F. Z. Sun, W. S. Chen, G. Y. Yang, S. J. Guo, Y. J. Li, X. L. Zhao, Y. Zhang, J. Sun, B. Fan, S. L. Yang and K. Li. 2003. Genetic diversity of microsatellite loci in fifty-six Chinese native pig breeds. Yi Chuan Xue Bao. 30(3):225-233.

Zhang Z. G., B. D. Li and X. H. Chen. 1986. Pig breeds in China. Shanghai Scientific and Technical Publisher, Shanghai, China.