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Genetic diversity of four protected indigenous chicken breeds in China using microsatellite markers

Lin Wei[1,2], Bin Chen[2#], Xiao-ying Li[1], Sheng-gui Liu[1] & Jing-jing Wang[2]
[1] The Department of Life Science, Huaihua University, Huaihua 418008, China
[2] College of Animal Science and Technology, Hunan Agriculture University; Key Laboratory of Hunan Province for Livestock and Poultry Breeds Improvement, Changsha 410128, China

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Abstract

The genetic diversity of four protected indigenous chicken breeds was evaluated with 25 microsatellite markers. Polymorphism information content (PIC), heterozygosity with the estimator of genetic differentiation F_{ST} and Nei's genetic distance were evaluated. The results showed that these four protected local chicken populations showed high levels of diversity. The proportion of inter-population subdivision among the four protected local chicken populations was 16.0%. The average heterozygosity was 0.514, 0.581, 0.567 and 0.589 in Dongan, Xuefeng black-bone, Xianghuang and Taoyuan chickens, respectively, while the average *PIC* estimates were 0.455, 0.581, 0.557 and 0.576. A phylogenetic tree was constructed using genetic distance and the neighbour-joining method. Its topology reflects the general pattern of genetic differentiation among the four chicken breeds. The results also showed high genetic diversity and genetic variation among all the breeds. The information about the four local breeds estimated by microsatellite analysis may be useful as an initial guide for the effective conservation of chicken genetic diversity and developing conservation strategies.

Keywords: Native chicken; genetic variation; microsatellite DNA
[#] Corresponding author: hnchenbin@163.com

Introduction

Chicken is one of the most widely distributed livestock in China. It plays a significant role as a source of income and high-quality protein for humankind. Indigenous chickens appear to possess enormous genetic diversity, especially in adaptive traits, and the ability to survive harsh conditions and under minimum feeding regimens (Qu *et al*., 2006). Successful preservation and utilization of these local types depend on accurate assessment of genetic diversity and genetic structure. Four indigenous chicken breeds, namely Dongan, Xianghuang, Taoyuan and Xuefeng black-bone chickens were included in the National Poultry Genetic Resource Directory, and play an important role in socio-economic development and ecological values in Hunan Province. In addition, their precious values as genetic resources are used for the genetic improvement of chicken breeds. Dongan are special meat-type chickens, and have good meat characteristics, high nutritional value and other excellent traits, such as yellow feathers, yellow skins and yellow shanks (Qu *et al*., 2006). The Xuefeng black-bone chicken is a meat and egg-type chicken, whose qualities include black meat, black bone, black beak and black feet, which are rich in nutritive and medicinal value, and were formed through long-term natural selection in the Xuefeng mountainous region in Hunan Province (Wei *et al*., 2008). Xianghuang and Taoyuan chickens are excellent meat and egg-type

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local chicken breeds, which come mainly from Changsha and Taoyuan counties in Hunan Province, respectively (Gao *et al*., 2008). So far, limited genetic diversity research has been conducted on these four chicken breeds, except for the study of Wei *et al.* (2008) on Xuefeng black-bone chickens.

Many molecular markers have become excellent means for the study of genetic variation (Chang *et al*., 2005; Chen *et al*., 2003), such as random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP), microsatellite DNA, and sequence-related amplified polymorphism (SRAP) (Zietkiewicz *et al*., 1994; Li *et al*., 2001). Among these DNA markers, microsatellites are widely used since they are numerous, randomly distributed in the genome, highly polymorphic, and with co-dominant inheritance (Groen *et al*., 1994; Kavaca *et al*., 1999). Many microsatellites have been mapped in chickens, and are used to study the genetic relationships among breeds (Tadano *et al*., 2007; Kaya *et al*., 2008). The purpose of this study is to estimate the level of genetic differentiation and phylogenetic relationships among the four indigenous chicken breeds in China. This information can contribute to the conservation and utilization of local chicken breeds.

Materials and Methods

Samples were obtained from 179 unrelated individuals, representing four indigenous chicken breeds in Hunan Province. These included 50 Dongan chickens (DA) from Dongan county, 50 Xianghuang chickens (XH) from Liuyang county, 29 Taoyuan chickens (TY) from Taoyuan county and 50 Xuefeng black-bone chickens (XF) from Hongjiang city. Blood samples (3 mL) were collected with syringes from the wing vein into a tube containing DNA preservation solution as an anti-coagulating agent. All samples were stored at −80 ºC for further analysis.

Genomic DNA was isolated from blood using a phenol/chloroform extraction method (Sambrook, 2002). The DNA was quantified with a spectrophotometer, comparing band intensities with known standards of DNA marker on 1.5% agarose gel. The working solution of DNA (approx. 25 ng/µL) was dissolved in sterile double-distilled water. In a preliminary experiment, 40 SSR primers were tested on four random individuals from each breed. Based on the amplification result, 25 microsatellite loci were further investigated, which were listed in Table 1. All primers were synthesized by ShenggongBio-Tech. Co., Ltd. Shanghai, China.

Polymerase chain reaction (PCR) amplifications were performed on PTC-200 thermal cyclers. A total reaction volume of 8 µL with 1 µL of 10 × buffer, 0.6 µL of 25 mmol $MgCl_2$, 0.2 µL of 10 mmol dNTPs, 0.1 µL of 5 U/µL *Taq* DNA polymerase, 0.3 µL of 10 pmol/µL each primer, and approximately 50 ng of genomic DNA were used. The reaction was carried out by initial denaturation at 94 ºC for 3 min, and then denaturing at 94 ºC for 30 s, annealing at the temperature optimized for each primer pair for 30 s and extending at 72 ºC for 30 s for 35 cycles, followed by an extra extension step at 72 ºC for 5 min. The optimized annealing temperatures of different primer pairs are listed in Table 1. The amplification products were separated by electrophoresis on 12% non-denaturing polyacrylamide gels and visualized by silver staining (Su *et al*., 2006). The images data were analysed with Kodak Digital Science ID Image Analysis Software.

Based on microsatellite genotyping and allele frequencies, the number of alleles, effective number of alleles (*Ne*), observed heterozygosity (*Ho*), (Nei, 1987) expected heterozygosity (*He*), and Wright's (1978) fixation index (*Fis*) were estimated using the computer software package PopGene version 1.31 (Yeh *et al*., 1997). Allele frequencies obtained from the microsatellite genotypes were used to calculate *PIC* (polymorphism information content) values (Botstein *et al*., 1980) using the computer software package Cervus 3.0 (Marshall *et al*., 1998; Kalinowski *et al*., 2007) in order to measure the information obtained by a microsatellite. Based on microsatellite genotyping, Nei's (1978) unbiased genetic distance between breeds was estimated. Software FSTAT (version 2.9.3.2) was used to test pairwise linkage equilibriums at all loci over any two groups to calculate the pairwise genetic differentiation F_{ST} (Weir & Cockerham, 1984). These results were used to construct phylogenetic trees by neighbour-joining cluster analysis with the appropriate options of computer software Mega Version 4.0 (Tamura *et al*., 2007).

Table 1 Primers used in the present study

Locus	primer's sequences(5'→3')	Annealing temperature (ºC)	Allele number	Fragment length (bp)	Chr.
ADL0188	F: CACTTCCAGTATTAACGTGA R: GTGGACACAATGAGTTCCTC	54	5	125–209	1
ADL0190	F: TCAGCTCTTCAGGCAAAAAG R: AACTTGGACCACAATCTTAT	52	5	220–231	2
MCW0224	F: ATTACCTTTCTTCATTAACGCC R: TTCATAGACTTGAGCGAGGAC	56	8	261–301	3
MCW0170	F: TTGTGAAACTCACAGCAGCTG R: TTATAGCAGGCTGGCCTGAAG	60	6	223–267	4
MCW0029	F: CATGCAATTCAGGACCGTGCA R: GTGGACACCCATTTGTACCCTATG	56	5	149–194	5
MCW0176	F: AAAGAGAAGTATAAAACATGCC R: TCCATTCTTGGCAGTGCATAG	58	7	251–278	6
MCW0120	F: CTATGTAAAGCTTGAATCTTCA R: ATTCCTGGGTGCTAATTTACC	54	6	250–287	7
ADL0121	F: CTGGAACAAGAGGGCTTTGC R: GGATGTGAAAAATCTCCTGG	56	7	125–157	8
MCW0134	F: GGAGACTTCATTGTGTAGCAC R: ACCAAAAGACTGGAGGTCAAC	56	6	260–284	9
MCW0035	F: CAGAAACATTTGGACTTGGCTT R: TTGCTTCATTTCTAGTCTCCAGTT	60	6	205–233	10
MCW0097	F: GGAGAGCATCTGCCTTCCTAG R: TGGTCTTCCAGTCTATGGTAG	56	6	263–309	11
MCW0198	F: GATCTTTGCTACCATCCACTG R: ACCCATCTGGTTGGACTATGC	58	4	294–324	12
MCW0104	F: TAGCACAACTCAAGCTGTGAG R: AGACTTGCACAGCTGTGTACC	56	5	189–263	13
LEI0098	F: AAAAGACAATGCAATTGGTGC R: CTGCCACTGATGCTGTCACT	60	7	147–170	14
MCW0080	F: GAAATGGTACAGTGCAGTTGG R: CCGTGCATTCTTAATTGACAG	58	6	278–337	15
MCW0330	F: TGGACCTCATCAGTCTGACAG R: AATGTTCTCATAGAGTTCCTGC	56	3	260–290	17
MCW0217	F: GATCTTTCTGGAACAGATTTC R: CTGCACTTGGTTCAGGTTCTG	56	6	153–174	18
MCW0094	F: GGAGCTGGTATTTGTCCTAAG R: GCACAGCCTTTTGACATGTAC	60	9	77–195	19
MCW0165	F: CAGACATGCATGCCCAGATGA R: GATCCAGTCCTGCAGGCTGC	55	4	125–144	23
MCW0285	F: AGTTGGAGGTTATATTA CGGG R: TATGACATAATCCACGCTGAG	58	5	156–300	26
MCW0328	F: ATGGAAACAGATGGAGCTGGC R: CTCCAATCCCAGGCTCCAAC	57	6	262–324	27
ADL0284	F: CAGAGTTCATCCGCCACTGC R: CCTCCCCACTAACATTGGAA	60	6	137–67	28
LEI0254	F: AGACCACTGGATCCAACTC R: GTCTGGAACTCATCCCTTCATC	55	6	85–101	Z
MCW0294	F: ACTGAACAGAAACAGTCTTCC R: CTTCTCTAGATGTCCACTACC	55	6	286–317	Z
MCW0154	F: GATCTGTTTTATCACACACAC R: CCATTTCCTTTGTTATCAGGC	55	6	161–193	Z

Note: F: forward primer; R: reverse primer.

Results

The genetic diversity and differentiation among the four local chicken breeds at the 25 microsatellite loci were estimated. The numbers of alleles per locus and the size range of alleles are listed in Table 1. All loci were polymorphic in the four breeds. The observed numbers of alleles varied from 3 (MCW0330) to 9 (MCW0094) and the mean number of alleles across all loci was 5.84. The observed heterozygosity *(Ho)* ranged from 0.268 (LEI0254) to 0.726 (LEI0098) (Table 3). The expected heterozygosity *(He)* was quite high, ranging from 0.448 (LEI0254) to 0.861 (MCW0224) (Table 2). The *PIC* among loci was highest for MCW0224 (0.854) and lowest for ADL0210 (0.447) (Table 2).

There was highly significant genetic divergence across the four breeds for every locus. The F_{ST} values ranged from 0.058 (MCW0330) to 0.243 (MCW0097). Using the multilocus F_{ST}, approximately 16.0% of the total genetic variation can be explained by breed differences, and the remaining 84.0% was owing to the differences among individuals (Table 2).

Table 2 Nei's estimation of heterozygosity at every locus average over breeds

Locus	Number of effective alleles(*Ne*)	Observed heterozygosity (*Ho*)	Expected heterozygosity (*He*)	Hetero-zygosity (*H*)	*Fst*	F_{IS}	*PIC*
ADL0188	3.046	0.575	0.673	0.586	0.128	0.016	0.647
ADL0190	3.675	0.480	0.729	0.594	0.170	0.034	0.722
MCW0224	7.074	0.569	0.861	0.649	0.243	-0.007	0.854
MCW0170	3.973	0.653	0.750	0.579	0.232	0.065	0.740
MCW0029	3.010	0.614	0.669	0.564	0.159	0.069	0.663
MCW0176	4.258	0.502	0.767	0.591	0.237	0.059	0.759
MCW0120	3.660	0.703	0.728	0.621	0.147	-0.029	0.711
ADL0121	3.792	0.631	0.738	0.618	0.157	-0.099	0.733
MCW0134	3.044	0.653	0.673	0.569	0.147	-0.024	0.659
MCW0035	4.665	0.631	0.787	0.700	0.105	-0.050	0.779
MCW0097	4.850	0.653	0.796	0.601	0.243	0.003	0.788
MCW0198	3.105	0.553	0.679	0.552	0.217	-0.024	0.644
MCW0104	3.010	0.525	0.669	0.586	0.152	0.038	0.661
LEI0098	3.716	0.726	0.733	0.611	0.171	0.018	0.725
MCW0080	2.904	0.676	0.657	0.548	0.165	0.047	0.641
MCW0330	2.382	0.659	0.581	0.537	0.058	0.058	0.502
MCW0217	3.770	0.631	0.736	0.577	0.202	0.052	0.727
MCW0094	5.587	0.670	0.823	0.642	0.216	-0.115	0.815
MCW0165	1.892	0.413	0.473	0.385	0.151	0.003	0.462
MCW0285	2.186	0.480	0.544	0.485	0.102	0.001	0.536
MCW0328	2.721	0.569	0.634	0.545	0.152	0.065	0.629
ADL0284	2.453	0.608	0.594	0.526	0.131	0.061	0.588
LEI0254	1.8104	0.268	0.448	0.388	0.162	-0.064	0.447
MCW0294	2.3892	0.553	0.583	0.532	0.088	0.016	0.577
MCW0154	2.135	0.525	0.533	0.490	0.068	-0.072	0.562
Mean (SD)	3.404 (1.22)	0.581 (0.101)	0.674 (0.106)	0.563 (0.071)	0.160 (0.010)	0.012 (0.022)	0.663

The estimated multilocus heterozygosities varied from H = 0.514 in DA to H = 0.589 in TY. The mean effective allele number was between 3.20 (XF) and 3.72 (TY). Among breeds, the mean *PIC* value was 0.455, 0.581, 0.557 and 0.576 for DA, XF, XH and TY, respectively. Wright's fixation index (*Fis*) values ranged from –0.16 (XF) to 0.14 (DA). Deviation from Hardy-Weinberg equilibrium (HWE)

between loci and breeds was tested with FSTAT (version 2.9.3, Goudet, 2001). Significance levels were adjusted using Bonferroni correction for multiple testing. Significant deviations (P <0.05) from HWE were observed at the breed level (DA) (Table 3).

Table 3 Within breed genetic variation

Breeds	Mean *He* for all loci	Mean effective number of alleles	Mean *PIC* for all loci	Mean *Ne* for all loci	*Fst*	Gene diversity
DA	0.514 (0.139)	3.24 (0.597)	0.455	2.218 (0.619)	0.221*	0.603±0.263
XF	0.581 (0.101)	3.20 (0.957)	0.581	2.527 (0.612)	0.128	0.514±0.211
XH	0.567 (0.150)	3.56 (0.711)	0.557	2.523 (0.670)	0.131	0.535±0.134
TY	0.589 (0.109)	3.72 (0.936)	0.576	2.592 (0.633)	0.160	0.547±0.125

*Significant deviation from HWE (P <0.001); DA: Dongan chicken; XF: Xuefeng black chicken; XH: Xianghuang chicken; TY: Taoyuan chicken.

Using Nei's (1978) unbiased genetic distance (Table 4) and the neighbour-joining method, a phylogenetic tree was constructed for the four chicken breeds. The smallest genetic distance, between Xianghuang and Taoyuan chickens, was 0.242. The largest genetic distance, between Dongan and Xuefeng black-bone chickens, was 0.507. The neighbour-joining dendrogramme in Figure 1 was drawn using the genetic distances given in Table 4. The Xianghuang, Taoyuan chickens and Dongan chickens and Xuefeng black-bone breeds were clustered as two groups, in order to support the reliability of this analysis.

Table 4 Genetic distance between breeds

Breed	DA	XF	XH	TY
DA	-			
XF	0.507	-		
XH	0.366	0.461	-	
TY	0.492	0.452	0.242	-

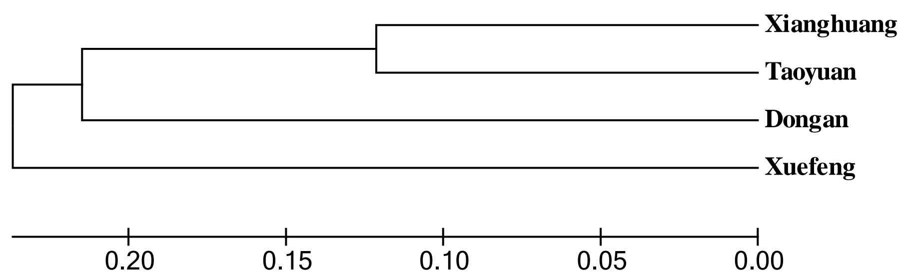

Figure 1 Neighbour-joining tree based on Nei's genetic distance.

Discussion

Choosing scientific microsatellite markers is essential to analyse genetic diversity. In this study, the 25 microsatellite markers with extensive coverage on main genome and high polymorphism worked very well for this purpose. They also demonstrated their utility as informative molecular markers in the four indigenous chicken breeds. The mean number of alleles for all loci was similar among the four breeds (Table 3). The mean effective number of alleles in this research for overall loci was 3.404 (Table 2). Compared with a previous study (Wei *et al*., 2008), the present research revealed the same microsatellite allele variation in Xuefeng black-bone chickens (*Ne*, 3.43). The result is similar to that of other studies. For instance, Emara *et al*. (2002) examined 41 microsatellite markers in three commercial broiler pure lines and reported an average number of alleles per marker of 3.5, 2.8 and 3.1 for each of the lines. Hillel *et al*. (2003) reported that the mean number of alleles was 3.5 within 52 breeds. Shahbazi *et al*. (2007) reported a mean number of alleles of 4.5 per locus in Iranian native chickens.

Heterozygosity estimates within the breeds were based on a set of markers showing substantial number of detected alleles and polymorphic information content. This result showed that genetic diversity in the four indigenous chicken breeds was high. A similar result (0.45 - 0.67) was reported by Wimmers *et al*. (2000) for African, Asian and South American local chickens. However, the mean *He* recorded in this research is lower than that reported by Zhang *et al*. (2002) in Chinese native chickens (0.63 - 0.86) and by Shahbazi *et al*. (2007) in Iranian native chickens (0.62 - 0.74). It was also higher than Hillel *et al*. (2003), who reported that the average gene diversity within 52 breeds across all 22 loci was 0.47. The variation of expected heterozygosity may be adduced to differences in location, sample size, breed structure and microsatellite markers. The high mean heterozygosity values may be attributed to the low level of inbreeding, low selection pressure and large number of alleles present in one breed. In this study, the high gene diversity value is a reflection of a high intra-breed genetic variation among these chicken breeds in Hunan province in China.

The mean PIC was an ideal index to measure the polymorphism of allele fragments. The mean PIC among loci was 0.663 (Table 2), and almost all markers were highly informative in the four indigenous chicken breeds. The F_{IS} represents a degree of nonrandom mating (deviation from HWE). A positive value for F_{IS} indicates deviation from HWE. In this study, causes of deviations from HWE, such as shortage of samples, selective mating, low levels of polymorphism, were not the major concerns in the breeds. The presence of inbreeding may be a reasonable explanation for the observed lack of agreement with HWE. Significant deviations from HWE were observed for 17 loci in Dongan chickens. These results indicate that the level of gene flow among breeds is restricted. The observed divergence probably reflects the human selection and the bottleneck effect (Maak *et al*., 2003).

The degree of genetic differentiation among these breeds and the high levels of significance for the inter-breed F_{ST} estimations indicate a relatively low gene flow among the four local chicken breeds and a relatively high reproductive isolation. The mean F_{ST} value of 0.16 indicates that approximately 16.0% of the total genetic variation is caused by breed differences, whereas the remaining 84.0% is due to differences among individuals within breeds. Chen *et al*. (2006) reported a mean F_{ST} value of 0.16 from 12 chicken breeds using 29 microsatellite markers, and Zhang *et al*. (2008) reported a mean F_{ST} value of 0.142 from seven chicken breeds using 29 microsatellite markers.

The genetic distance among the four protected chicken breeds varied from 0.242 to 0.507, and the mean genetic distance between any given breeds was 0.42, reflecting that these breeds are genetically isolated from each other. Hillel *et al*. (2003) emphasized that genetic distance measures based on gene frequencies were in good agreement with the genetic diversity of these breeds, indicating that these approaches fit the history of domesticated chickens well. The genetic differentiation found among the four protected local chicken breeds in the neighbour-joining dendrogramme (Figure 1) was confirmed by their breeding origin and evolution.

The information about the four protected local chicken breeds estimated by microsatellite analysis may be useful as an initial guide to defining objectives for designing future investigations of genetic variation and developing conservation strategies. Microsatellite data in this study indicate that the four protected local chicken breeds showed a high within-breed genetic variation, which is a favourable factor

when planning conservation and improvement programmes. The effective conservation of genetic diversity in the chicken gene pool relies essentially on the understanding of genetic diversity patterns of chicken breeds in a conservation region, including the levels and distribution of the diversity (Chen *et al.*, 2006). It is particularly important to conserve the chicken's genetic diversity on farm management (or on-farm conservation), because the combination of farmers' diverse needs with the breeds in different ecosystems has created and accumulated wide genetic variation. This study revealed a high genetic diversity within the four protected local chicken breeds. The results from this study indicated that the genetic variation of chicken breeds is still remarkably rich. The considerably rich genetic diversity of chicken breeds in China can be attributed to its complicated local geographical conditions where different farming practices and agro-ecosystems exist. The diversity may also be significantly associated with its rich culture diversity that promotes miscellaneous needs and applications of chicken breeds. Relative isolation of the various areas has probably played a considerable role in reducing the exchange with modern improved chicken breeds. Such a factor has played an essential role in maintaining the genetic diversity of Chinese chicken breeds. In addition, it is important to carry out more studies in future, which can provide us with useful information for the effective conservation of chicken genetic diversity and a roadmap for conservation strategies of chicken genetic resources.

Conclusion

These four protected indigenous chicken breeds throughout Hunan Province in China are resources of considerable socio-economic value. Their genetic diversity was evaluated with 25 microsatellite markers in this study. The result demonstrated that these breeds showed high polymorphism. A phylogenetic tree was constructed using genetic distance and the neighbour-joining method. The four breeds were clustered as two groups, in order to support the reliability of this analysis. The information about the four local breeds that was estimated by microsatellite analysis may be useful as an initial guide to defining objectives for designing future investigations of genetic variation and developing conservation strategies

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