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Differentiation and Phylogenetic Relationship of Different Geographic Populations of Tibetan Chicken, *Gallus gallus domesticus*

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Abstract.- The Tibetan chicken is a breed protected by the Chinese state. It mainly comprises a Tibet population and a Sichuan population. This study aims to evaluate genetic diversity, phylogenetic relationships and population differentiation of populations through microsatellite and phenotypic methods. The results of 12 microsatellite loci reveal 62 alleles in all populations, 29 alleles shared by two populations, 23 private alleles in the Tibet population, and 10 private alleles in the Sichuan population. The mean expected heterozygosity (He) is 0.635 and 0.496 for the Tibet and Sichuan populations, respectively; the mean observed heterozygosity (Ho) is 0.457 and 0.371 for the Tibet and Sichuan populations, respectively. F_{is} values of Tibetan and Sichuan populations are 0.333 and 0.163, respectively. F_{st} value between these populations is 0.2172 (P<0.0001). Phenotypic research indicates that the Sichuan population has better growth performance and smaller body size than that of the Tibet population. Thus, the two populations of Tibet chicken have a medium level of gene differentiation and different phenotypic characters, and should be separated protection.

Keywords: Tibetan chicken, geographic population, microsatellite, genetic diversity, phylogenetic relationship

INTRODUCTION

Tibetan chicken (*Gallus gallus domesticus*) is a special breed of poultry that was listed as the first-class key species of animals by agriculture ministry of China (Yang *et al*., 2010; Ji *et al*., 2001). Tibetan chicken mainly distributes in the whole area of Tibet Autonomous Region, northwest region of Sichuan Province on the Qinghai-Tibet plateau including 8 counties such as Batang, Daocheng, Xiangcheng, Jiulong, Ruo er-gai, Songpan, Ma er-kang and De ge (Wu *et al*., 2011a,b). Because Tibetan chicken still inhabits in the special Qing-Tibetan plateau with low pressure of artificial selection due to the local primitive living style of the minority, it keeps the strong flight ability, body appearance as the red jungle fowl, and a close genetic relationship with the red jungle fowl (Bao *et al*., 2005, 2008). Tibetan chicken lays 60-90 eggs per year, and the average weight of adult chicken is less than 1500g. Hence its productive performance is lower than other chicken breeds in China. In recent years, with the continuously development of the west region of China, some commercial chicken breeds from domestic and foreign companies have been introduced into Qing-Tibet plateau. The introgression of other chicken breeds into purebred Tibetan chicken populations has caused a decline in population and distribution of this species (Bao *et al*., 2008). Moreover, degradation caused by inbreeding was found in the habitat because the poor management results of the shortage of manage means and knowledge (Li *et al*., 2010). All of above mentioned made Tibetan chicken became endangered.

During the past decade, genetic investigations of the Tibetan chicken have mainly focused on the diversity of Tibetan chicken brought from Tibet habitat (Du *et al*., 2004; Zhai *et al*., 2010), genetic relationships between Tibetan chicken and other indigenous poultries (*e.g.*, Bao *et al*., 2008; Zhai *et al*., 2010; Li *et al*., 2010) , the capability of adapting plateau (Lu *et al*., 2011; Wang *et al*., 2007) and protecting suggestion (Ji *et al*., 2001; Wu *et al*., 2011a,b). However, to our knowledge, almost no research has examined the diversity of Tibetan chicken in Sichuan population and its relationships to the Tibetan population. The Tibetan habitat has some difference compared with the Sichuan habitat. Firstly, the average altitude of the Tibet habitat is above 4,000m compared with 3,000m to 4,000m in Sichuan habitat, which results in lower air temperature in Tibet habitat than that in Sichuan habitat (Du *et al*., 2009) and the annual average

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temperature is 0°C in Tibetan habitat and 11.5-12.8°C in Sichuan habitat. Secondly, a lower annual average precipitation exists in Tibet habitat than that in Sichuan habitat (Wu *et al*., 2011a,b), which makes a higher average relative humidity in Sichuan habitat than that in Tibet habitat (Du *et al*., 2009). Furthermore, these two main original habitats of Tibetan chicken are relatively isolated regions without convenient transportation (Zhai *et al*., 2010). Obviously, study and understanding the diversity and their relationship of different Tibetan chicken populations are essential for conservation and breeding development.

It is well known that microsatellites are suitable for analyzing genetic diversity and phylogenetic relationship among closely related populations (Buchanan *et al*., 1994; MacHugh *et al*., 1998), and the technique has been widely applied to chicken breeds (Jossi *et al*., 2003; Weigend and Romanov, 2001; Romanov and Weigend, 2001; Akram *et al.,* 2010). Therefore, in this paper, we use microsatellite markers to analyze the genetic diversity and phylogenetic relationship of the Tibet and Sichuan populations of Tibetan chicken and investigate some growth performance parameters. Moreover, we also analyze phenotype differences between these populations.

MATERIALS AND METHODS

Samples

Muscle tissue was sampled from a total of 79 Tibetan chicken adults. Of these 79 individuals, 29 individuals were sampled from a breeding base of Tibetan chickens in Tibet (Linzhi County) and the remaining individuals from a breeding base of Tibetan chickens in Sichuan Province (Ganzhi County). The breeding base in Linzhi County was the foundation seed farm with 800 chickens from core protected area, and Ganzhi County foundation seed farm with 1000 chickens from core protected area.Any two samples among these individuals did not come from one founder.

Genomic DNA was isolated using a standard proteinase K digestion and phenol-chloroform extraction procedure (Sambrook *et al*., 1989).

In order to analyze phenotypic characters between the two populations, 140 individuals from Linzhi County base (Tibet population) and 50 individuals from Ganzhi County base (Sichuan population) were used to analyze growth, adult body size and body weight.

Microsatellite genotyping

Twelve polymorphic microsatellite loci (Table I) originally designed for the Tibetan chicken (Yang *et al*., 2009, 2010) were used to analyze the genetic structure of Tibetan chickens under previously published conditions (Yang *et al*., 2009, 2010). Five loci were trinucleotide repeats, and the others were dinucleotides. All loci have been mapped in the genome of *Gallus gallus* breed Red Jungle fowl and located on 8 chromosomes (Table I).

The dye-labeled PCR products (with 5'-FAM) of all microsatellite primers were separated on an ABI 3100 DNA sequencer following the protocol provided by the sequencer. Fragment lengths were assigned using GeneScan software (ABI) and a GeneScan-500 [Tamra] size standard.

Data analysis

A Bayesian clustering method was used to infer population structure, as well as to identify migrant or admixed individuals based upon the software STRUCTURE 2.3.3 (Pritchard *et al*., 2000; Falush *et al*., 2003; Hubisz *et al*., 2009). We performed a series of independent runs using population clusters (K) from 1 to 8, assuming the burn-in and replication values set at 60,000 and 10^6, respectively, and an admixture model, in STRUCTURE (Pritchard *et al*., 2000; Falush *et al*., 2003; Hubisz *et al*., 2009). We ran three independent simulations for each K value and the independent runs produced consistent results for the same K value.

The programme CERVUS 2.0 (Marshall *et al*., 1998) was used to determine heterozygosity estimates, polymorphic information content (PIC) and number of alleles. Allelic richness (*AR*) and Fis was calculated with the FSTAT 2.9.3 program package (Goudet, 2001). Allele frequency and private alleles were analyzed using the software CONVERT 1.31 (Glaubitz, 2004).

GENEPOP 4.0 (Rousset, 2008) was used to test the genotypic distribution for conformance with Hardy-Weinberg equilibrium (HWE) and linkage

disequilibrium (LD). All probability tests were based on the Markov chain method using 1000 de-memorization steps, 100 batches and 1000 iterations per batch.

An assessment of population pairwise comparisons was derived from ARLEQUIN 3.1 and the statistical significance of Fst was tested with 10000 permutations as implemented in ARLEQUIN 3.1 (Excoffier *et al*., 2005).

To detect the genetic signature of a population decline, the program BOTTLENECK was applied (Piry *et al*., 1999). The Wilcoxon sign-rank test was used to determine significance.

The software SPSS11.5 was used to compare Tibetan chicken's growth, adult body size and weight between Tibet and Sichuan populations.

RESULTS

Assignment test

Assignment tests showed the highest log likelihood value of the data (Ln probability) obtained when K = 2 (Fig. 1), which showed a significant genetic differentiation between the Tibet and the Sichuan populations. A significant difference (P<0.0001) was showed between the two populations when all individuals were considered on the statistical significance of F_{st} in ARLEQUIN 3.1.

Based on the assignment test, some individuals with admixed ancestry origin (8 individuals from the Tibet population and 18 individuals from the Sichuan population) existed in the two populations (Fig. 1). Based on pedigree analysis, these individuals did not come from the same breeder and their founders did not come from one place. The two habitats of Tibetan chicken were relatively isolated regions without convenient transportation (Zhai *et al*., 2010). These individuals with admixed ancestry origin might be introduced by human and might underestimate the genetic differentiation between the two populations. The following analysis did not include these individuals.

Genetic diversity and Bottleneck analysis

In total, 62 alleles were identified for the 12 microsatellite loci (Table I), including 23 private alleles in Tibet, 10 private alleles in Sichuan and 29 alleles shared from both the Sichuan and Tibet

Table I.- Characterization of microsatellite loci for Tibetan chickens in Sichuan and Tibet populations, including number of alleles observed (Na) for 5 microsatellite loci in the two populations, chromosome location (Chr) of *Gallus gallus* breed Red Jungle fowl, repeat motif structure (motif), locus name, number of individuals were genotyped (N), number of alleles (A), observed heterozygosity (Ho), expected heterozygosity (H_E), Polymorphic information content (PIC), HWE *P*-values and allelic richness (A_R).

Locus	Motif	Chr	Tibet							Sichuan						
			Na	N	A	Ho	H_E	PIC	P-values	A_R	N	A	Ho	H_E	PIC	P-values	A_R
22-F6	$(TTG)_n$	1	4	21	4	0.857	0.751	0.685	0.1609	4	32	3	0.594	0.576	0.498	0.8568	3
H5-4	$(TG)_n$	27	3	21	3	0.095	0.553	0.437	0.0000	2.993	32	2	0.125	0.119	0.110	1	1.976
10-A5	$(TTG)_nCTG(TTG)_m$	1	4	21	2	0.762	0.502	0.37	0.0261	2	32	3	0.5	0.508	0.390	0.6747	2.594
6-A9	$(TTG)_n$	4	4	21	3	0.286	0.591	0.51	0.0071	3	32	2	0.313	0.411	0.323	0.2063	2
B12-6	$(AC)_n$	2	6	21	5	0.452	0.619	0.557	0.2107	4.836	32	4	0.423	0.498	0.413	0.1160	3.685
19-B2	$(TTG)_n$	2	7	19	6	0.158	0.812	0.761	0.0000	6	32	3	0.281	0.546	0.468	0.001	2.999
H7-2	$(AC)_n$	7	4	21	4	0.362	0.563	0.529	0.0617	4	32	3	0.316	0.418	0.398	0.243	3
9-B2	$(TTG)_n$	3	5	21	5	0.442	0.627	0.614	0.4216	4.906	32	2	0.416	0.534	0.446	0.726	2
C8-1	$(AC)_nGC(AC)_m$	1	5	21	4	0.359	0.724	0.516	0.0714	3.995	32	3	0.219	0.568	0.327	0.0452	2.856
H11	$(AC)_n$	12	7	21	6	0.687	0.624	0.705	0.3217	6	32	4	0.468	0.715	0.526	0.2157	4
D11-5	$(TG)_nGC(TG)_m$	1	7	21	5	0.534	0.632	0.652	0.0812	4.912	32	3	0.362	0.427	0.422	0.0931	3
F12	$(TG)_n$	14	6	21	4	0.487	0.617	0.525	0.1325	4	32	4	0.429	0.629	0.520	0.106	3.817
Average	-	-	-	-	4.3	0.457	0.635	0.572	-	4.220	-	3	0.371	0.496	0.403	-	2.911

populations. The number of alleles per locus ranged from three at locus MberH5-4 to a maximum of 7 at locus MberH11, Mber19-B2, and MberD11-5 (Table I). Compared to genetic diversity indices for the Sichuan population, a significant higher genetic diversity was found in the Tibet population based on the higher average number of allelic richness (Table I, ANOVA, $P = 0.003$).

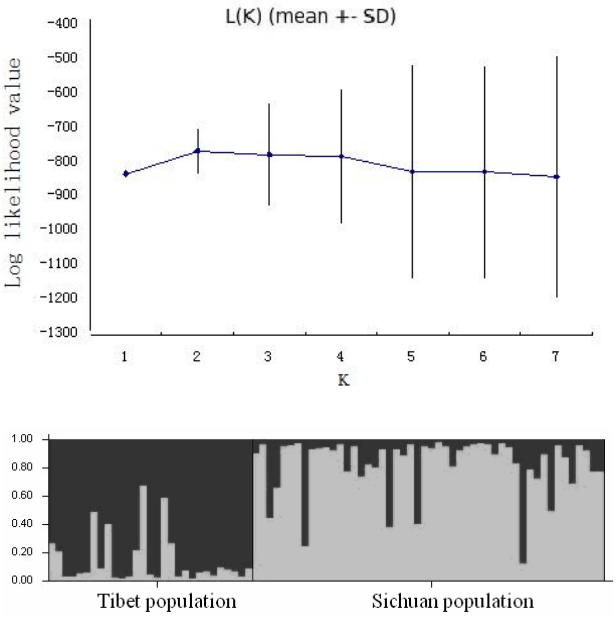

Fig. 1. Top: Structure assignments, Plot displays mean log-likelihood values (LnP(D)) for 7 independent runs for each value of K for K =1–7. The highest value was at K = 2.
Below: Bayesian Clustering Analyses through the software STRUCTURE (admixture model, burn-in and replication values set at 50000 and 10^6, respectively). Each individual was represented by a thin vertical bar, which is partitioned into 2 colored segments that represent the individual affiliation to each of 2 clusters.

A moderate degree of heterozygosity was observed in this study. The mean expected heterozygosity (He) was 0.635 (ranging from 0.502 to 0.812) in Tibet, as compared to 0.496 (ranging from 0.119 to 0.715) in Sichuan. The mean observed heterozygosity was 0.457 in Tibet and 0.371 in Sichuan (Table I). No significant variation of the observed heterozygosity and a significant variation of the expected heterozygosity (ANOVA, P=0.011) existed between the two populations (Table I).

Populations experiencing a recent bottleneck would display a reduction in allele number and reduced heterozygosity, the latter manifesting itself more slowly (Cornuet and Luikart, 1997). No evidence for recent bottlenecks in these two populations was found based on the Wilcoxon sign-rank test (P<0.01) using the infinite allele model (IAM), two-phase model (TPM) or stepwise mutation model (SMM).

Linkage disequilibrium (LD) and Hardy-Weinberg equilibrium

Following Bonferroni correction, highly significant LD tests were not shown for any pair of loci within each of the populations. Hardy-Weinberg equilibrium (HWE) tests revealed that four loci (MberH5-4, Mber10-A5, Mber6-A9, and Mber19-B2) in the Tibet population (P<0.05) and one locus in the Sichuan population (P = 0.001, Mber19-B2) deviated significantly from HWE (Table I) for significant heterozygote deficiency (P<0.01). MberC8-1 in Sichuan population deviated slightly from HWE (Table I) for slight heterozygote deficiency (P = 0.0452).

Population differentiation analysis

The results indicate a middle level of genetic differentiation between the Tibet and Sichuan populations with an Fst-value of 0.2172 (P<0.0001). This result was supported by assignment test (Fig. 1) and the phenotypic characters between the two populations. However, inbreeding exists in the two populations. Fis-value was 0.333 for Tibet population and 0.163 for Sichuan population.

Our datum on growing development show the Sichuan population has a better performance compared with that in the Tibet population. For example, the weight of the Sichuan population was larger than that of Tibetan population at 30d,90d,120d,150d (ANOVA, P<0.05) (Table II). Similar results were observed for the weight of adult too (Table III). Moreover, the chest depth of the Sichuan population was smaller than that of Tibet population (ANOVA, P<0.05) (Table III). The body length, chest breadth, width of pelvis and phalanx

length of the Sichuan population were different from the Tibetan population, but not significantly. No microsatellite allele or genotypes in this study correlated with one of the growing development index.

DISCUSSION

Due to the geographic isolation between the Tibet population and the Sichuan population (Zhai *et al*., 2010), it was impossible to exchange gene between the two populations in natural conditions. The individuals with admixed ancestry orgins in this study might be introduced by human. These individuals can underestimate the reliable genetic differences between the Tibet and the Sichuan populations and it is necessary to refuse these individuals when the differences between the two populations were compared. Assignment test with microsatellite loci could be used to estimate the proportion of an individual's genome that was derived from one or the other populations (Pritchard *et al*., 2000) and has been widely used in identifying some hybridizations in a recent past time, including the introgression of the domestic cat (*Felis catus*) into Scottish wild cat (*Felis silvestris*) gene pools (Beaumont *et al*., 2001) and some hybridizations between wolves and dogs (Andersone *et al*., 2002). In this study, the assignment test was used to determine these individuals with admixed ancestry origin (Fig. 1). In order to protect the unique genetic characters of different Tibet chickens populations, these individuals with admixed ancestry origin should not be considered as core breeding individuals.

In our study, only 4 loci in Tibet population and 1 locus in Sichuan population were deviated to Hardy-Weinberg equilibrium (HWE) tests (Table I) for significant heterozygote deficiency. Moreover, Tibet population has a higher F_{is} value than that of Sichuan population. These results show that Tibet population has higher inbreeding level than Sichuan population. Thus, enhancing genetic management will increase the genetic diversity of the two populations.

Assignment tests showed that a significant genetic differentiation between the Tibet and the Sichuan populations (Fig. 1), which was supported

Table II.- The growth indicators of Tibetan chicken, including the average monthly weight(AMW, gram), and the relatively growth rate(RGR) of Tibetan chicken in male(M) and female(F) of Sichuan and Tibet population.

		Dates	0 Month	1 month	2 month	3 month	4 month	5 month	6 month
Male	Sichuan	AMW (g)	30.72±1.16*	127. 8±17.65*	258.92±32.13	636.92±78.7*	879.41±140.38*	1278.57±162.57*	1312.5±153*
		RGR	—	316.02	102.60	177.42	38.07	45.3	2.65
	Tibet	AMW (g)	28.02±1.03	82.85±17	239.25±27	436.96±58.7	622.04±73	771.61±103.9	969.57±137.5.74
		RGR		195.68	188.77	121.37	42.36	24.05	25.66
Female	Sichuan	AMW (g)	29.86±0.97*	115.21±15.85*	218.92±29.65	531.67±94.43*	773.08±107.27*	1012.5±118.94*	1110.42±194.77
			122.58±20.65						
		RGR	—	285.83	90.02	131.57	45.41	30.97	9.67
	Tibet	AMW (g)	27.41±1.01	75.70±17.40	203.31±27.19	352.83±47.7	494.71±74.2	610.04±90.1	765.30±133.234
		RGR		173.62	168.57	78.75	40.21	23.31	25.45

[1]Value =Mean + SD; * means P<0.05

RGR=(W_{i+1}- W_i)/ W_i×100%, in which W_{i+1} means the (i+1)th month's weight(gram), W_i. means the ith month's weight

Table III.- The body size and body weight of Tibet chicken adults.

	Sichuan (n-50)		Tibet (n=140)	
	Male	**Female**	**Male**	**Female**
Weight (g)	1520.05±335**	1220.67±290	1162.50±160.2	923.49±138.31
Body length (cm)	20.85±1.000	18.24±1.9	18.7±1.345	16.82±0.914
Chest depth (cm)	10.63±1.3**	9.53±1.28	14.70±2.264	10.60±1.81
Chest breadth (cm)	6.67±0.716	6.01±0.55	7.27±0.8	6.67±0.75
Width of pelvis (cm)	7.3±0.88	6.67±0.59	8.3±0.54	7.5±0.20
Phalanx length (cm)	9.4±0.425	7.78±0.20.	9.24±0.3	7.86±0.49

[1]Value =Mean ± SD; * means P<0.05

by the F_{st}-value of 0.2172 (P<0.0001) obtained from ARLEQUIN 3.1. The differentiation between the Tibet population and the Sichuan population was larger than that among red jungle fowls and Chinese domestic fowls. Bao *et al*. (2008) reported that in the whole population, the average of genetic differentiation among red jungle fowls and Chinese domestic fowls, measured as F_{st} value, was 16.7% (P<0.001). Moreover, the phenotypic characters (Tables II, III) between the two populations also supported the result originated from genetic data. The Sichuan population's growing development shows a better performance compared with that in Tibet. For example, the weight of the Sichuan population was about 500-600g, whereas, that was only about 300g in Tibet at 90 days. Moreover, the weight of Sichuan population was significant larger than that in Tibetan population. In addition, the body size of the Sichuan population was smaller than that in Tibet. Thus, the two Tibet chicken populations should be considered as two separate conservation units.

Moreover, a high genetic diversity existed in the two populations of Tibetan chicken, including high allelic richness (4.220 for the Tibet population and 2.911 for the Sichuan population) and high heterozygosity (Table I). This result corroborates other works (Du *et al*., 2004; Li *et al*., 2010). The level of genetic diversity of Tibet chicken population was close to the chicken populations that reported by Granevitze *et al*. (2007) who found that within chicken populations, the average observed heterozygote frequency was 0.46, with a range between 0.20 and 0.64. Our study also showed that the Tibet population contained higher mean genetic diversity than the Sichuan population (Table I). However, the Tibet population has a higher Fis value than that of the Sichuan population, which showed a higher lever of inbreeding in the Tibet population than in the Sichuan population. Thus, in order to keep the genetic diversity of the Tibet population, more precise genetics management should be paid on the Tibet population than on the Sichuan population. Genetic monitoring should be used to identify genetically distinct individuals and more mating chances should be given to the individuals who contain rare alleles.

CONCLUSIONS

In this study, Tibet chickens contain a high genetic diversity and didn't experience a bottleneck. The two populations of Tibet chicken have a medium level of gene differentiation, which was supported by phenotypic characters. These results suggested that Tibet chicken should be considered as two independent geographic populations. Therefore, we should separately protect the gene diversity and character differentiation of the different geographic population.

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