Investigation of PCR-RFLPs within Major Histocompatibility Complex *B-G* Genes Using Two Restriction Enzymes in Eight Breeds of Chinese Indigenous Chickens

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ABSTRACT : New polymorphism of major histocompatibility complex *B-G* genes was investigated by amplification and digestion of a 401bp fragment including intron 1 and exon 2 using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique with two restriction enzymes of *Msp* I and *Tas* I in eight breeds of Chinese indigenous chickens and one exotic breed. In the fragment region of the gene, three novel single nucleotide polymorphisms (SNPs) were detected at the two restriction sites. We found the transition of two nucleotides of A294G and T295C occurred at *Tas* I restriction site, and consequently led to a non-synonymous substitution of asparagine into serine at position 54 within the deduced amino acid sequence of immunoglobulin variable-region-like domain encoded by the exon 2 of *B-G* gene. It was observed at rare frequency that a single mutation of A294G occurring at the site, also caused an identical substitution of amino acid, asparagine 54-to-serine, to that we described previously. And the transversion of G319C at *Msp* I site led to a non-synonymous substitution, glutamine 62-to-histidine. The new alleles and allele frequencies identified by the PCR-RFLP method with the two enzymes were characterized, of which the allele *A* and *B* frequencies at *Msp* I and *Tas* I loci were given disequilibrium distribution either in the eight Chinese local breeds or in the exotic breed. By comparison, allele *A* at *Msp* I locus tended to be dominant, while, the allele *B* at *Tas* I locus tended to be dominant in all of the breeds analyzed. In Tibetan chickens, the preliminary association analysis revealed that no significant difference was observed between the different genotypes identified at the *Msp* I and *Tas* I loci and the laying performance traits, respectively. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 7 : 942-948)***

Key Words : Chicken, *B-G* Gene, PCR-RFLP, SNP, Nonsynonymous Substitution

INTRODUCTION

Chicken major histocompatibility complex (MHC), initially identified as B blood group system (Briles et al., 1950; Schierman and Nordskog, 1961, 1963, 1964), is composed of two regions, the B region and the *Rfp*-Y region that segregated independently each other (Miller et al., 1994; Juul-Madsen et al., 1997; Pharr et al., 1998) and located on a microchromosome 16 (Miller et al., 1996). The MHC B (region) contains three tightly linked parts, *B-G*, two *B-F* (*F* I and *F* IV genes) and *B-LB* (*L* I and *L* II genes) loci (Briles et al., 1993; Miller et al., 1994; Pharr et al., 1998), where the *B-G* genes, determined as class IV by comparison with the MHC of mammals, is unique to chicken breeds (Miller et al., 1991).

The highly polymorphic *B-G* antigens (a cell surface antigens) encoded with *B-G* genes have been confirmed to have an adjuvant activity in humoral response to the B system class I antigens (Hala et al., 1981) and to antigens of other blood group system (Schierman et al., 1967). Especially, the B-G immunoglobulin Variable-region-like (Ig-V-like) domain (a single extracellular domain) might act as an important role in immune system functions of antigen presentation and recognition (Hunkapiller et al., 1989). The full length of *B-G* cDNA sequence and the genomic organization and sequence variation were characterized in B^{21} chicken embryo (Miller et al., 1991). Jarvi et al. (1999) identified *B-G*-like genes in cranes. After that, the clones of exons 1 and 2 of *B-G* gene were generated by PCR from genomic DNA samples of Camperos broiler chickens and genetic variability within the *B-G* gene family was revealed by single stranded conformational polymorphism (SSCP) assays (Iglesias et al., 2003).

Chinese native chicken breeds are the most important component of the world animal resources and most of them have some special and unique characteristics because of the extremes of climate and geography contributing to the development of the breeds. It has been attracting many researchers to investigate the genetic diversity and structure of different native breed populations (Cheng et al., 2000). Their goals were to search for proper way to utilize the good characteristics of local chickens. However, the genetic polymorphism of *B-G* genes in Chinese indigenous breeds was rarely reported.

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Table 1. The characters and historical background of the eight Chinese indigenous chickens sampled

Breed	Main characteristics	Geographical locations	Domesticated before
Tibetan chicken	Meat and egg type; adapting to plateau area at altitude between 2, 200 and 4, 100 meters;	Tibet Autonomous Region and West of Sichuan province	Unclearly
Silkies	Medical type; high medicine value of meat;	Jiangxi and Fujian province	More than 1,000 years
Langshan chicken	Meat and egg type; excellent meat taste and rich nutrients, good body gain;	Jiangsu province	Unclearly
White earlobes	Egg type; relatively high egg production;	Jiangxi and Zhejiang province	Unclearly
Gushes	Meat and egg type; relatively high egg production;	Henan and Anhui province	Unclearly
Xianju chicken	Egg type; adapting to extremely sultry weather;	South of Zhejiang province	More than 400 years
Beijing fatty chicken	Meat and egg type; excellent meat taste and rich nutrients; good body gain;	Beijing city	More than 250 years
Chinese game chicken	Game type;	Henan and Shandong province	3,000 years

The breeds in the table were mainly protected poultry genetic resources in China, and the breeding characteristics were reported by Chen (2004).

In this study, the genetic polymorphism in *B-G* gene was investigated by amplification of the 401 bp fragment spanning over parts of intron 1 and exon 2 encoding the Ig-V-like domains using PCR, followed by cloning, sequencing and restriction fragment length polymorphism analysis with two restriction enzymes of *Msp* I and *Tas* I in eight breeds of Chinese indigenous chickens and one commercial broiler breed. Then the association between the different genotypes at the *Msp* Ⅰ and *Tas* I loci and the laying performance traits was analyzed in Tibetan chicken.

MATERIALS AND METHODS

DNA samples and PCR amplification

A total of 381 indigenous Chinese chicken from eight different native breeds (Tibetan chicken, Langshan chicken, Silkies, Xianju chicken, Gushi chicken, White earlobes (Buff Baier) chicken, Beijing Fatty chicken and Chinese Game chicken) and commercial broiler chickens were used. The characters and historical background of the native chickens sampled were given in Table 1. For each bird, about 1 ml blood was collected from the wing vein and mixed with 10 ml 10 mM EDTA (pH 8.0) containing 10 mM Tris-HCl（pH 8.0）and 2% SDS. Genomic DNA used in PCR amplification was isolated from blood using the procedure as described by Briles et al. (1993).

Specific PCR primers (Forward: 5'- TGT CTC TTC TTC ACC TCC ACC-3', Reverse: 5'- GCA GTT CTG TTC TCC CTT CAT-3') used to amplify a 401bp fragment that spanned over parts of intron 1 and exon 2 of MHC *B-G* gene were designed based on a published chicken *B-G* gene sequence (AF388372). The primers were synthesized by Shanghai BioAsia Biotechnology Co. Ltd.

PCR products were amplified on a PTC-100™ programmable thermal controller (MJ Research, Inc.). The PCR reactions were performed in a volume of 20 μl consisting of 50-60 ng of chicken genomic DNA, 1×PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM each primer and 1 U Taq DNA polymerase (MBI Fermentas). The PCR conditions were at 95°C for 5 min followed by 35 cycles of a denaturing at 95°C for 30 s, an annealing at 60°C for 30 s, an extension at 72°C for 30 s, and a final extension at 72°C for 4 min. The products were visualized by electrophoresis on 2% agarose gels stained with 0.25 μg/ml ethidium bromides.

PCR-RFLP analysis

The PCR products were subsequently digested with restriction enzymes *Msp* I and *Tas* I (MBI Fermentas) to identify the polymorphism of MHC *B-G* gene, respectively. A volume of 4 μl PCR product was digested with 5 U restriction enzyme of *Msp* Iat 37°C or with Tas I at 65°C according to the methods employed by Chu et al. (2003) and Padma et al. (2004). The digested products were analyzed on a 3% agarose gel stained with 0.25 μg/ml ethidium bromides at 70 V for 1-2 h and the gel was visualized under UV-transilluminator and finally documented by the gel photography system (GeneSnap from SynGene).

Cloning of PCR products and sequencing

PCR products were purified with Wizard prep PCR purification system (Pregma, Madison, WI, USA), and cloned into Pregma pGEM-T easy vector according to the method published by Sambrook et al. (2001). The fragment obtained was sequenced by a commercial service. For further sequence analysis, identical sequences were obtained from two independent PCR amplifications from the same bird. Then the sequences were analyzed with BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

Statistical analysis

The following statistical model was used to analyze the relationship between the genotypes at *Msp* I and *Tas* I loci

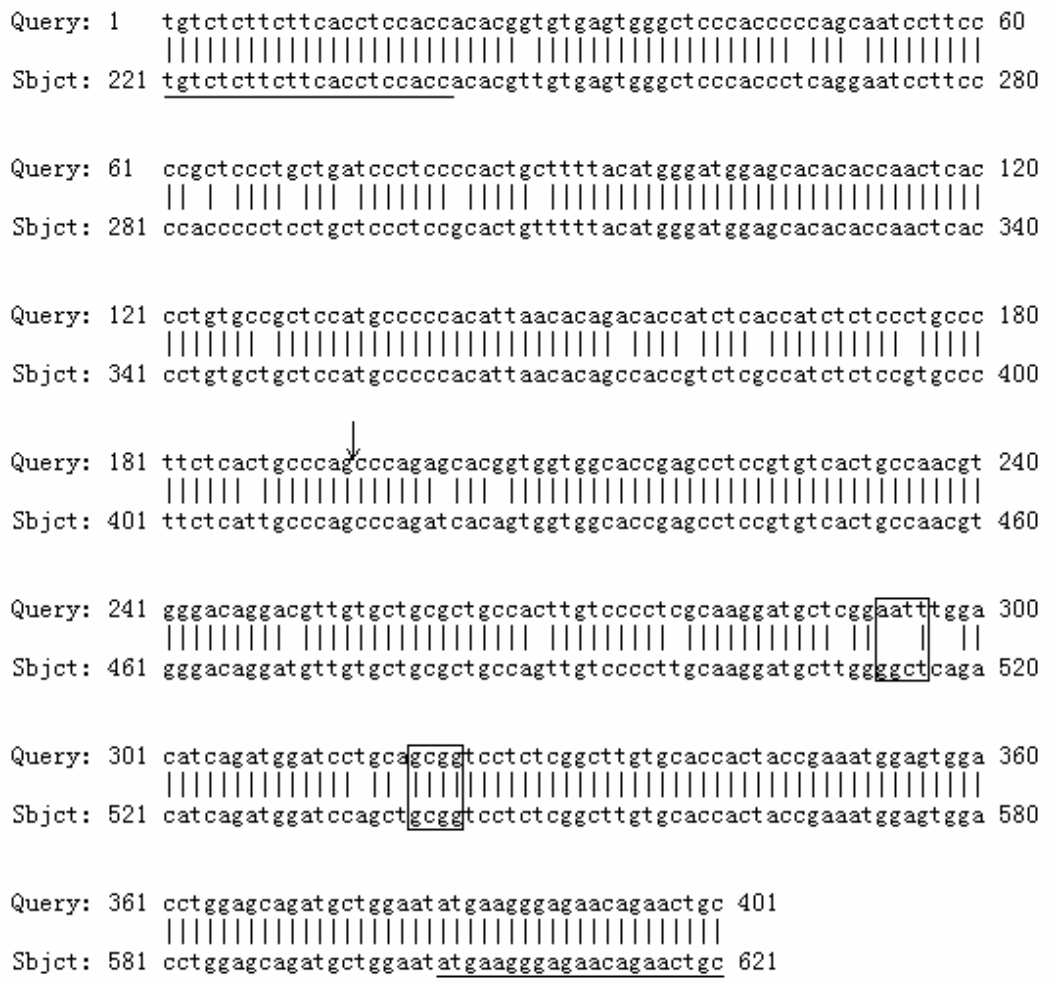

Figure 1. Alignment of the amplified fragment (an allele sequence, GenBank accession No. AY744340) at the top obtained from Tibetan chicken to the corresponding sequence of *B-G* gene (Accession No. AF388372). The nucleotide sequence of each primer binding region is underlined; the arrow shows the boundary between intron 1 and exon 2 of *B-G* gene; and the two boxes indicate the positions of *Tas* I (^AATT) and *Msp* I (C^CGG) sites within *B-G* gene, respectively.

of the *B-G* gene and laying performance traits using GLM procedure of SPSS11.0.

$$Y_{ijk} = \mu+\alpha_i+\beta_j+e_{ijk}$$

Where, Y_{ijk} is phenotypic value of the target trait, such as laying number, μ is population mean, α_i is the fixed effect of the i^{th} genotype at *Msp* I locus within the *B-G* gene ($i = 1, 2$); β_j, the fixed effect of the j^{th} genotype at *Tas* I locus ($j = 1, 2, 3$) and e_{ijk} is random error effect of each observation.

The records of laying performance (including laying number, egg weight and body weight) were from 166 individuals, which were randomly picked from eight hundreds of indigenous Tibetan hens that were hatched and labeled at the same time and raised in same conditions of management and nutrition on the farm of Agriculture and Animal Husbandry College of Tibetan University. Approaching 32 weeks of age (age of laying starting), each hen was raised per cage when the raising condition was maintained constantly. The number of eggs per layer in 210 days of laying cycle from 32 to 62 weeks of age was recorded day by day. During this period, 7 eggs per layer were randomly picked and weighed monthly, and the body weight was recorded with restriction of feed and water at 50 weeks of age in Tibetan hen.

RESULTS

Analysis of the nucleotide sequence amplified

A 401 bp PCR fragment was amplified with genomic DNA samples and the cloned PCR products from the chickens using the designed specific primers. The fragment obtained was sequenced by a commercial service. Then comparisons of the predicted sequences with the corresponding sequence in GenBank (AF388372) were done using the BLAST2 software provided by the NCBI server (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html)

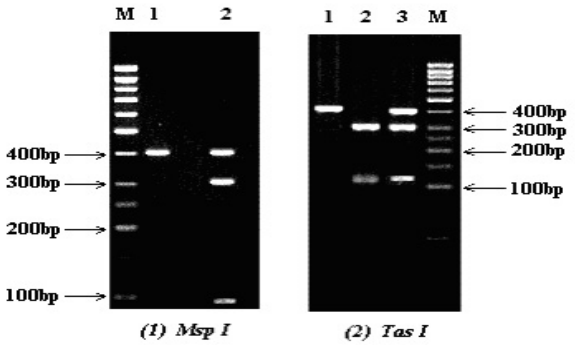

Figure 2. PCR–RFLP patterns of MHC *B-G* gene by *Msp* I and *Tas* I restriction enzymes in chicken; M: 50 bp DNA ladder; No.1-2 denotes the observed genotypes of *AA* and *AB* at *Msp* I locus; No.1-3 denotes the observed genotypes of *AA*, *AB* and *BB* at *Tas* I locus, respectively.

to assure that the expected sequences of chicken MHC *B-G* gene were isolated.

It was shown in Figure 1 that each primer binding region was completely identical to the objective DNA sequence in the GenBank, and the PCR product size of the 401 bp fragment within MHC *B-G* gene was corresponding to the expected sequence that spanned over inton 1 and exon 2. All sequences were identified by comparing the sequences from cloned PCR products with those of the direct genomic PCR products at same individual. For all birds, no more than two allelic sequences were observed, suggesting that the primer pair specifically amplified a single locus, and no breed-specific sequence was found in different individuals tested from the nine chicken breeds, showing the universality of the primers used to amplify the fragment of MHC *B-G* gene.

PCR-RFLP analysis and allele frequencies

Novel polymorphisms (Figure 2) in the 401 bp fragment of MHC *B-G* gene were detected with restriction enzyme *Msp* I and *Tas* I, respectively. At *Msp* I locus, a biallelic polymorphism was found, the restriction enzyme cut the 401 bp fragment into two portions. Allele *A*, in which the polymorphic restriction site at position 320 is absent, is characterized by the presence of the fragment of length 401 bp, while for allele *B*, which possesses the polymorphic restriction site, this fragment is cut into two fragments of 319 bp and 82 bp showing on the gel. Hence, three expected genotypes, *AA* (401 bp), *AB* (401 bp, 319 bp and 82 bp) and *BB* (319 bp and 82 bp), would be detected. However, only two genotypes of *AA* and *AB* were found in all the chicken breeds analyzed but in the Langshan chicken, single genotype (*AA*) was observed. At *Tas* I locus, two alleles were observed. Allele *A*, in which the polymorphic restriction site at position 293 is absent, showed only one band (401 bp length) in the gel, while for allele *B*, which possesses the polymorphic restriction site, this fragment is cut into two fragments of length 292 bp and 109 bp showing on the gel. So, three genotypes, *AA* (401 bp), *AB*

Table 2. Frequencies of allele and genotype at the *Msp*I locus of MHC *B-G* gene in Chinese indigenous chicken breeds

Breed	Number	Genotype frequency			Allele frequency	
		AA	AB	BB	A	B
Tibetan chicken	190	0.6579	0.3421	0	0.8289	0.1711
Silkies	26	0.1538	0.8462	0	0.5769	0.4231
Langshan chicken	17	1.000	0	0	1.0000	0
White earlobes	30	0.7333	0.2667	0	0.8667	0.1333
Gushes	19	0.1579	0.8421	0	0.5789	0.4211
Xianju chicken	18	0.5556	0.4444	0	0.7778	0.2222
Beijing fatty chicken	28	0.8571	0.1429	0	0.9286	0.0714
Chinese game chicken	13	0.6923	0.3077	0	0.8462	0.1538
Commercial broiler	28	0.2857	0.7143	0	0.6429	0.3571
Average frequencies*		0.6276	0.3724	0	0.8138	0.1862

* Means the average frequencies of allele and genotype in the eight Chinese indigenous breeds (except for the commercial broiler).

Table 3. Frequencies of allele and genotype at the *Tas*I locus of MHC *B-G* gene in Chinese indigenous chicken breeds

Breed	Number	Genotype frequency			Allele frequency	
		AA	AB	BB	A	B
Tibetan chicken	166	0.1325	0.4699	0.3976	0.3675	0.6325
Silkies	20	0.1000	0.4000	0.5000	0.3000	0.7000
Langshan chicken	20	0	0.3000	0.7000	0.1500	0.8500
White earlobes	24	0	0.0833	0.9167	0.0417	0.9583
Gushes	22	0	0.9091	0.0909	0.4545	0.5455
Xianju chicken	24	0	0.9167	0.0833	0.4583	0.5417
Beijing fatty chicken	24	0.2500	0.1667	0.5833	0.3333	0.6667
Chinese game chicken	12	0	0.4167	0.5833	0.2083	0.7917
Commercial broiler	27	0.1111	0.6667	0.2222	0.4444	0.5556
Average frequencies*		0.0962	0.4647	0.4391	0.3285	0.6715

* Indicates the same meaning to Table 2.

(401 bp, 292 bp and 109 bp) and *BB* (292 bp and 109 bp), were detected in Tibetan chicken, Silkies, Beijing fatty chicken and commercial broilers. But only two genotypes, *AB* and *BB*, were observed in the other breeds.

The frequencies of allele and genotype at the *Msp* Ⅰ and *Tas* I loci of the exon 2 of MHC *B-G* gene estimated in the nine populations were given in Table 2 and 3, respectively.

Association of the genotypes with laying performance in Tibetan chicken breed

As shown in Table 4, no significant difference was observed between the different genotypes and the traits analyzed at the *Msp* Ⅰ and *Tas* I loci. But the layers with genotype *AA* had more laying number than those of genotype *AB* at *Msp* Ⅰ locus. As for the *Tas* I locus, the birds with genotype *AA* had more laying number than those of genotype *AB*, and *AB* genotype had more laying number than those of genotype *BB*.

From Table 5, we found no significant difference was observed in egg weight and body weights of 50 weeks of age in the different genotypes we analyzed at the two loci. But the layers with genotype *AA* had greater average egg weight than those with genotypes *AB* and *BB* at the *Tas* Ⅰ locus, while, the layers with genotype *AB* had greater average body weight at 50 weeks of age than those with genotypes *AA* and *BB* at the locus. At *Msp* Ⅰ locus, layers with genotypes *AA* and *AB* had very similar values estimated in the two group traits.

DISCUSSION

By sequence alignment, it showed the sequence obtained had high homology (93-96%) to the corresponding fragment of *B-G* gene from Camperos broiler chickens identified by Iglesias et al. (2003). All the sequences detected shared the common conserved region and variable region patterns that were similar to the aforementioned literature data. For each bird, the sequence analysis either using direct PCR product or the clone of PCR product of genomic DNA from the same animal gave no more than two alleles, furthermore, by comparison the pattern types of PCR-RFLP observed in same bird, no variation appeared in all the detected chicken breeds in the condition. Taken together, we concluded that the designed primer pairs and the PCR conditions used in the study could be used to specifically, and sensitively amplify the alone, expected sequence of *B-G* gene. Moreover, the pattern types of the PCR-RFLP detected in the present study were universal and could be used for typing across chicken breeds.

In this research, there are three novel SNPs within the MHC *B-G* gene were detected, which occurred correspondingly at the two restriction sites of *Msp* Ⅰ (C^CGG) and *Tas* Ⅰ (^AATT), respectively. At the *Msp* Ⅰ site (320 bp), the transversion of G319C led to the non-synonymous substitution, glutamine→histidine at position 62 of the deduced amino acid sequence of Ig-V-like domain (Miller et al., 1991) encoded by exon 2 of the MHC type antigen *B-G* (accession number: AAP23070) gene. In fact, besides the aforementioned change of the codon at position 62, another substitution A318T was also detected, this change led to a codon for leucine which was identical to the result reported by Iglesias et al. (2003)

At *Tas* Ⅰ site (293bp), It's more frequency that the transition of the two nucleotides of A294G and T295C

Table 4. Association between the different genotypes at *Msp* I and *Tas* I loci within the *B-G* gene and laying numbers in the six laying phases of Tibetan hens

Locus	Genotype (*N*)	60 days ($\overline{X}$ ±*SE*)	90 days ($\overline{X}$ ±*SE*)	120 days ($\overline{X}$ ±*SE*)	150days ($\overline{X}$ ±*SE*)	180 days ($\overline{X}$ ±*SE*)	210 days ($\overline{X}$ ±*SE*)
Msp I	*AA* (121)	17.54±1.070	32.46±1.895	44.33±3.140	54.83±4.012	62.17±4.692	68.58±5.013
	AB (45)	16.42±1.848	30.58±3.173	42.17±4.226	53.00±4.764	61.33±5.566	68.33±5.807
Tas I	*AA* (22)	18.27±1.668	34.18±2.299	46.72±3.575	58.18±4.447	65.91±5.785	73.55±6.526
	AB (78)	17.29±0.765	32.94±1.270	44.51±1.906	56.16±2.284	64.35±2.571	70.24±2.699
	BB (66)	16.17±1.23	31.76±1.760	44.21±2.789	55.33±3.712	63.61±4.474	70.09±4.812

N means number of different genotypes, *AA*, *AB* and *BB*, respectively. $\overline{X}$ is least square mean, *SE*, standard error.

Table 5. Association between the different genotypes at *Msp* I and *Tas* I loci within the *B-G* gene and egg weight and body weight at 50 weeks of age in Tibetan hens

Locus	Genotype	Egg weight (g)		50 week body weight (kg)	
		N_1	($\overline{X}$ ±*SE*)	N_2	($\overline{X}$ ±*SE*)
Msp I	*AA*	847	46.84±1.003	121	1.33±0.7240
	AB	315	46.67±0.8236	45	1.29±0.2013
Tas I	*AA*	154	49.53±1.768	22	1.30±0.0671
	AB	546	46.17±0.6710	78	1.38±0.0666
	BB	462	46.98±1.195	66	1.28±0.1061

N_1 means the number of eggs detected in the experiment; N_2, the number of birds examined.

happened together, the mutations led to the non-synonymous substitution of amino acid, asparagine 54-to-serine, within the Ig-V-like domain. It's rare to see that an alone mutation, A294G, occurred at the site, which also caused change of a codon for asparagine into a condon for serine. But we haven't found that an alone mutation occurred at position 295 of the restriction site. If the last one happened, it would only lead to a synonymous substitution of amino acid within the domain of the *B-G* antigen. In this study, it wasn't observed that an A→G substitution at position 293 changes a codon for serine into a codon for glycine as detected in the domain (Iglesias et al., 2003), this discrepancy was due to the large difference from origins of the breeds analyzed. This result conformed that the exon 2 sequence of *B-G* gene is highly polymorphic. The polymorphic *B-G* genes are associated with the immune-system functions of antigen presentation and recognition (Hunkapiller et al., 1989; Miller et al., 1991). Nucleotide variations in encoding region usually play very important role in effect on the molecular structure of *B-G* antigen, and consequently affect its specificity and immune function. The implication of high variability in the immunoglobulin-like domains is a possible function in anticipatory immune defense (Iglesias et al., 2003). The SNPs in Ig-V-like domain of *B-G* gene may directly determine the immunoglobulin-like nature of the extracellular domain of *B-G* antigen molecule, which could lead to variation in disease resistance and susceptibility.

From the distribution of allele and genotype frequencies estimated at the two loci within *B-G* gene detected, we found that allele *A* at *Msp* Ⅰ locus was tended to be dominant in all of the breeds we analyzed, while, the frequency of allele *A* was very low (average 0.1862). No genotype *BB* was observed at the site. Why the distributions in allele and genotype frequencies at the locus were so disequilibrium? It may be due to several reasons. (1) The number of animals in each population we detected is not enough to demonstrate the true event. As the result, an extreme allele frequency was estimated. (2) The allele *B* was inferred be tightly linked either with a lethiferous, detrimental or disadvantageous allele or with an unfavorable trait. Hence, the homozygotes with *BB* genotype either couldn't survive or were eliminated under natural selection pressure or competition from the environment to which they were adaptive (Koshy á et al., 1998). (3) The allele *B* was considered to be a newly arrival member to response to a common pathogen (Bodmer, 1972; Takahata and Nei, 1990). The reason why it was so rare in frequency may come from what it was of relatively too recent origin.

However, the allele *B* was tended to be dominant at *Tas* I locus, meanwhile, the frequency of allele *A* was very low (average 0.3285). And rare genotypes *AA* were found at the site (average frequency 0.0962). This situation was very similar to the distribution of allele and genotype frequencies estimated at the *Msp* Ⅰ locus examined above. This result could be explained by referring to the aforementioned hypotheses.

Many studies have demonstrated that chicken MHC association with particular productivity traits such as egg production, egg weight and body weight (Bacon 1987; Abplanalp et al., 1992; Cahaner et al., 1996; Lakshmanan et al., 1997). In this preliminary study, in order to find a potential valuable molecular marker of selection for higher laying intensity and egg weight in marker-assisted selection (MAS) program, we tentatively tried to search for the association of the novel polymorphisms by two restriction enzymes of *Msp* Ⅰ and *Tas* I with the laying performance traits detected. But we found no significant difference was observed between the traits and different genotypes at the *Msp* Ⅰ and *Tas* Ⅰ loci in Tibetan chicken. This result indicated that the two loci within the *B-G* gene were neither causative mutations nor disequilibrium linkage with the causative changes or the target traits we analyzed. To further confirm the suggestion increasing the number of birds and records from different populations and chicken breeds will be required

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