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Association between Polymorphisms of MSTN and MYF5 Genes and Growth Traits in Three Chinese Cattle Breeds

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ABSTRACT : The objective of this study was to assess the association of polymorphisms in *MSTN* and *MYF5* genes with growth traits in three Chinese cattle breeds. Only one homozygous animal with *BB* genotype at *MSTN* locus was observed in Jiaxian population which was at Hardy-Weinberg disequilibrium ($p<0.05$). The frequencies of allele *A* at *MSTN* locus and allele *B* at *MYF5* locus in the three Chinese breeds were 0.9550/0.9730/0.9720 and 0.8275/0.7581/0.7523, respectively. Allele *A* at *MSTN* locus and allele *B* at *MYF5* locus were dominant in these three populations. No statistically significant differences in growth traits were observed between the genotypes of the Jiaxian breed at *MSTN* and *MYF5* loci and the Nanyang breed at *MYF5* locus. However, there were statistically significant differences between the genotypes at *MSTN* locus of the Nanyang breed for WH, HG, HGI and HGBLR ($p<0.05$), and of the Qinchuan breed for BLI ($p<0.05$). The SNP in *MYF5* had significant effects on WH and HHC of Qinchuan animals ($p<0.05$). These results suggest that *MSTN* and *MYF5* are strong candidate genes that influence growth traits in cattle. Other SNPs of *MSTN* and *MYF5* or other linked genes should also be studied, which could lead to the development of selection plans to improve the performance of Chinese cattle and also promote the breeding of genuine beef cattle in China. (**Key Words :** Cattle, *MSTN*, *MYF5*, Growth Traits)

INTRODUCTION

Nanyang, Qinchuan, and Jiaxian cattle are three of the eight best cattle breeds in inland China. Due to their uniqueness, these breeds have been designated as nationally protected resources. They have been the representative of Chinese cattle breeds for their good performance traits and especially fleshy characteristics. For thousands of years, Nanyang, Qinchuan and Jiaxian breeds, famous for their large body size and excellent farming ability, have been the main labor force in agriculture and an important beef source. However, with the development of science and technology and the ever-increasing mechanization of farming, these good farming abilities have become obsolete. Compared with imported commercial beef cattle breeds, these three breeds have several drawbacks, such as an underdeveloped hind hip, slower growth rate and poor milk production. In an effort to adapt to the development of a commercial economy, it has become necessary for these three breeds to improve meat performance while maintaining their other excellent inherent characteristics.

Several candidate genes affecting muscle mass in farm animals may be selected on the basis of their participation in the processes of muscle development. Myostatin (MSTN), or growth and differentiation factor 8 (GDF8), is a member of the transforming growth factor β (TGF-β) super-family, which includes proteins that mediate key events in cell growth and development through signal transduction. Myostatin acts as a negative regulator of myogenesis (Lee and McPherron, 2001) and inhibits myoblast proliferation during the cell cycle and myogenic differentiation (Thomas et al., 2000; Taylor et al., 2001; Rios et al., 2002; Langley et al., 2002; Lu et al., 2007). In the absence of myostatin, the skeletal musculature of mice is two to three times greater in mass than that of wild-type mice (McPherron et al., 1997). Several cattle breeds are characterized by double muscling phenotype (an increase in the number of normal-sized

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Table 1. The primer sequences, position, PCR fragment sizes and the annealing temperatures of two genes

Genes	Primer sequence	Position*	Size (bp)**	Annealing temperature
MSTN	5´-CCCTACAGAGGCCACTTCAA-3´	9,142	1,346	63°C
	5´-CTCGCTGTTCTCATTCAGATC-3´	11,486		
MYF5	5'-GATAGCTGGCTGTGAATGAT-3'	966	1,190	60°C
	5' -CTGGCAACTGGGGAGAGAGAAG-3'	2,155		

* The position of the primers was established based on Genbank sequence AF348479 and M95684.

** Expected size of the fragments after amplification according to the same sequences.

muscle fibers that result in enlarged muscles with deep creases) caused by six different loss-of-function mutations at the *MSTN* locus (Grobet et al., 1997; Kambadur et al., 1997; Karim et al., 2001). Homogeneous and heterogeneous individuals with the mutations showed enlarged musculature, increased birth weight and greater growth rate (Casa et al., 1999; 2004). Although a large number of alleles of the gene have been identified in cattle, most are silent or neutral in their resultant effect. In some breeds double muscling is not associated with any disruptive mutation in the gene. Muscle fiber formation takes place during embryonic development and is regulated by the *Myo*D gene family, which consists of four genes, *Myo*D1 (*MYF*3), *myogenin* (*MYO*G or *MYF*4), *MYF5* and *MYF*6 (*MRF*4). MYF5, in combination with MYOD1, determines the muscular lineage and is the first factor of this family to be expressed in the embryo (Braun et al., 1989; 1990). Expression of *MYF5* in adult skeletal muscle is restricted to the satellite cell population (Beauchamp et al., 2000) and to the muscle spindles (Zammit et al., 2004). Knockout mouse experiments showed that *MYF5* and *MYO*D1 do affect muscle development, although they are redundant to a certain extent. Mice lacking both *MYF*5 and *MYO*D1 myogenic regulator factors were born alive but they died soon after birth (Rudnicki et al., 1993). *MYF*5 gene was mapped at bovine chromosome 5 region (0 to 30 cM) which was identified as having significant associations with the growth traits (Crosse et al., 1999; Li et al., 2002a, b; 2004). Because of its functions and support from the results of QTL studies, *MYF5* is considered as a candidate gene for growth related traits in meat producing animal species.

The objective of this study was to analyze the genetic variations of *MSTN* and *MYF5* genes in Nanyang, Qinchuan and Jiaxian cattle breeds and to investigate their corresponding effects on the growth traits of these three Chinese cattle breeds.

MATERIALS AND METHODS

Animal and data source

Four hundred and eleven female animals (Nanyang, 210; Qinchuan, 93; Jiaxian, 108) were used in this study. The animals originated from three farms. The Nanyang animals were from the breeding center of Nanyang cattle (Nanyang city, Henan Province, China), the Jiaxian animals were from the breeding farm of Jiaxian cattle (Jiaxian county, Henan Province, China) and the Qinchuan animals were from the reserved farm (Weinan city, Shaanxi Province, China). The Nanyang and Jiaxian animals were housed individually in the farms under strictly standardized conditions. The Qinchuan animals were born in 2006. The Nanyang animals were all of the same age and growth traits were measured every six months from six to twenty-four months old according to methods of Chen (1998). Growth traits measured in the Nanyang population were: birth weight, body weight (BW), hucklebone width (HBW), withers height (WH), heart girth (HG), body length (BL), body length index (BLI), heart girth index (HGI) and ratio of heart girth and body length (RHGBL). Only phenotypic data of Qinchuan animals aged four months and Jiaxian animals aged twenty-four months were available. Growth traits of the Qinchuan and Jiaxian animals measured were: WH, BL, HBW, HG, BLI, HGI, RHGBL, height at hip cross (HHC), rump length (RL), and hip width (HW).

DNA preparation and PCR amplification

Genomic DNA of 411 animals was isolated from 2% heparin-treated blood samples and stored at -80°C, following standard procedures (Sambrook et al., 2002). Using available sequence information from bovine *MSTN* (Genbank accession number AF348479) and bovine *MYF5* (Genbank accession number M95684), PCR primers were designed. PCR amplification was performed in 25 μl reactions containing 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 μM each of the four dNTP, 5 pmol of each primer and 1 U of Taq DNA polymerase (TaKaRa, China) under the following conditions: one cycle 94°C 3 min; 39 three-step cycles 94°C 30 s, the appropriate temperature 40 s, and 72°C 1 min followed by a last extension for 10 min at 72°C. The primer sequences, position, PCR fragment sizes and the annealing temperatures are listed in Table 1.

PCR-RFLPs analysis

Enzyme digestion was carried out in a total volume of 20 μl reaction mixture using *Dra*I, *Hae*III, *Hin*fI, *Hin*dIII, *Hpa*I, and *Taq*I according to product manuals. The products were analysed on ethidium bromide-stained 3% agarose gel and allele frequencies were estimated. Significant departure from Hardy-Weinberg equilibrium was tested by a x^2 test.

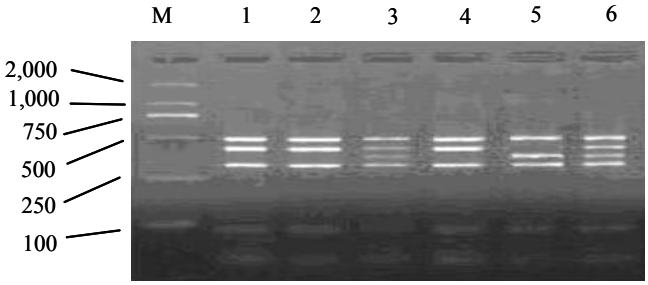

Figure 1. Agarose gel electrophoresis (3%) of PCR fragment of *MSTN* gene digested with *Dra*I. Genotype AA is in lanes 1, 2, 4, genotype AB is in lanes 3, 6, genotype BB is in lane 5. M is the D2000 marker.

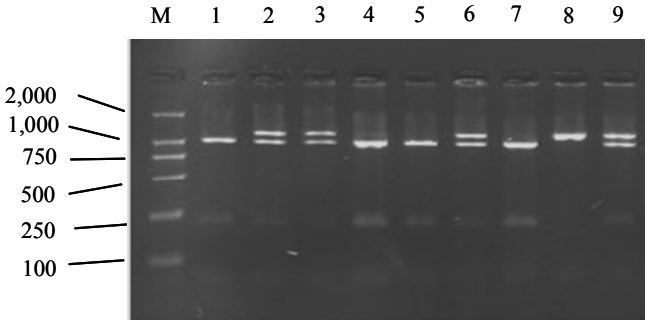

Figure 2. Agarose gel electrophoresis (3%) of PCR fragment of *MYF5* gene digested with *Taq*I. Genotype AA is in lane 8, genotype BB is in lanes 1, 4, 5, 7, genotype AB is in lanes 2, 3, 6, 9. M is the D2000 marker.

Statistical analysis

Data of the breeds were analysed by ANOVA (SAS software GLM procedure) using the following model:

$$Y_{ijklmn} = \mu + Breed_i + Age_j + Marker_k + (Breed \times Marker)_l + (Breed \times Age)_m + (Marker \times Age)_n + e_{ijklmn}$$

to estimate the effects of markers on growth traits; where Y_{ijklmn} is the observation of the trait, μ is the least square mean, $Breed_i$ is the effect of breed, Age_j is the effect of age, $Marker_k$ is the effect of marker genotype, $(Breed \times Marker)_l$ is the interaction of breed and marker, $(Breed \times Age)_m$ is the interaction of breed and age, $(Marker \times Age)_n$ is the interaction of marker and age and ε_{ijklm} is the residual effect.

RESULTS

Polymorphism/alleles frequencies

Two polymorphisms, *MSTN*-*Dra*I and *MYF5*-*Taq*I were observed after products were digested with enzymes. The polymorphism of *MSTN*-*Dra*I is caused by T/A transversion at position -371 (relative to ATG start codon) that introduces a site for *Dra*I restriction enzyme (Crisa et al., 2003). Digestion of the PCR fragment of *MSTN* promoter with *Dra*I resulted in fragment lengths of 505, 427, 321, and 93 bp for phenotype *AA*, 505, 365, 321, 93, and 62 bp for phenotype *BB* and 505, 427, 365, 321, 93 and 62 bp for phenotype *AB* (Figure 1). The frequency of allele A was dominant in all three Chinese cattle breeds. Only homozygous BB individuals were present in the Jiaxian population (Table 2). The genotype frequencies of *MSTN* in the Nanyang and Qinchuan populations agreed with Hardy-Weinberg equilibrium, whereas those in the Jiaxian population were at Hardy-Weinberg disequilibrium ($p<0.05$).

The *MYF5*-*Taq*I is a SNP (A/G) at the 1,948 bp position of intron 2 (Genbank accession number M95684) (Li et al., 2004). PCR-RFLP testing by digestion of a 1,190 bp amplicon with *Taq*I produced fragments of 1,190 bp in homozygous *AA* animals, 983 and 207 bp in homozygous *BB* animals, and 1,190, 983, 207 bp in heterozygous *AB* animals (Figure 2). The frequencies of the two alleles in the three breeds are given in Table 2. The frequency of allele *B* was dominant in the three breeds and individuals with *AA* genotype were less frequent than individuals with other genotypes. The genotype frequencies of *MYF5* in the three populations all agreed with Hardy-Weinberg equilibrium.

Association of polymorphism with growth traits

No statistically significant differences at *MSTN* locus were observed between the *AA* and *AB* genotypes of the Jiaxian breed concerning growth traits (Table 4). However, there were statistically significant differences between the genotypes of the Nanyang breed for the following growth traits ($p<0.05$) (Table 3): WH at eighteen-months, *AA* was significantly higher than *AB*; HG, HGI and HGBLR at six-months, *AA* was significantly lower than *AB*. In the Qinchuan breed, the individuals with genotype *AB* at *MSTN*

Table 2. Allele and genotype frequencies at *MSTN* and *MYF5* genes in three Chinese cattle breeds

Breeds	Individuals	Genes	Genotype frequencies			Allele frequencies		Locus equilibrium x^2 test
			AA	*AB*	*BB*	*A*	*B*	
Nanyang	210	*MSTN*	0.9100	0.0900	0.0000	0.9550	0.0450	Equilibrium ($p>0.05$)
		MYF5	0.0400	0.2650	0.6950	0.1725	0.8275	Equilibrium ($p>0.05$)
Qinchuan	93	*MSTN*	0.9460	0.0540	0.0000	0.9730	0.0270	Equilibrium ($p>0.05$)
		MYF5	0.0430	0.3978	0.5592	0.2419	0.7581	Equilibrium ($p>0.05$)
Jiaxian	108	*MSTN*	0.9351	0.0556	0.0093	0.9629	0.0371	Disequilibrium ($p<0.05$)
		MYF5	0.0360	0.4234	0.5406	0.2477	0.7523	Equilibrium ($p>0.05$)

Table 3. Least squares means, and standard errors for the effect of *MSTN* and *MYF5* genes on growth traits in Nanyang cattle

Ages	Growth traits	Genotypes at *MSTN* gene		Genotypes at *MYF5* gene	
		AA	*AB*	*AB*	*BB*
Birth	Body weight (kg)	31.333±0.867	29.743±0.256	29.962±0.541	29.949±0.335
Six months	BW (kg)	159.680±1.878	169.333±6.355	160.115±6.124	159.941±3.713
	HBW (cm)	18.379±0.215	18.333±0.726	18.308±0.426	18.235±0.263
	WH (cm)	106.117±0.499	107.444±1.687	106.577±0.900	105.897±0.556
	HG (cm)	128.748^{a}±0.838	134.444^{b}±2.821	128.500±1.748	129.235±1.060
	BL (cm)	105.544±0.740	108.556±2.504	105.654±1.540	105.618±0.934
	BLI	0.995±0.005	1.011±0.017	0.991±0.010	0.997±0.006
	HGI	1.214 ^{a}±0.006	1.252 ^{b}±0.019	1.206±0.011	1.221±0.007
	HGBLR	1.221 ^{a}±0.005	1.238 ^{b}±0.018	1.217±0.010	1.224±0.006
Twelve months	BW (kg)	222.794±2.289	221.000±7.707	223.280±6.124	223.779±3.713
	HBW (cm)	20.729±0.215	20.667±0.726	20.620±0.434	20.624±0.263
	WH (cm)	114.088±0.501	114.333±1.687	114.600±0.917	113.941±0.556
	HG (cm)	141.088±0.838	145.667±2.821	141.200±1.748	141.500±1.060
	BL (cm)	116.892±0.740	119.556±2.504	116.640±1.540	117.485±0.934
	BLI	1.024±0.005	1.045±0.017	1.017±0.010	1.031±0.006
	HGI	1.237 ^{a}±0.006	1.275 ^{b}±0.019	1.232±0.012	1.242±0.007
	HGBLR	1.209±0.005	1.221±0.018	1.211±0.011	1.207±0.006
Eighteen months	BW (kg)	297.373±3.129	297.000±10.532	292.760±6.124	301.706±3.713
	HBW (cm)	23.240±0.215	22.500±0.726	22.900±0.434	23.184±0.263
	WH (cm)	121.284^{a}±0.501	120.778^{b}±1.687	120.800±0.917	121.059±0.556
	HG (cm)	156.029±0.838	156.444±2.821	154.880±1.748	156.588±1.060
	BL (cm)	129.461±0.740	129.444±2.504	129.000±1.540	129.735±0.934
	BLI	1.069±0.005	1.072±0.017	1.068±0.010	1.071±0.006
	HGI	1.288±0.006	1.295±0.019	1.282±0.012	1.294±0.007
	HGBLR	1.206±0.005	1.210±0.018	1.201±0.011	1.208±0.006
Twenty-four months	BW (kg)	366.951±4.034	365.333±13.581	360.240±6.124	373.044±3.713
	HBW (cm)	25.407±0.215	24.778±0.726	25.020±0.434	25.353±0.263
	WH (cm)	126.225±0.501	126.667±1.687	126.080±0.917	126.603±0.556
	HG (cm)	168.642±0.838	172.000±2.821	167.520±1.748	169.390±1.060
	BL (cm)	138.127±0.740	137.778±2.504	137.320±1.540	138.485±0.934
	BLI	1.094±0.005	1.088±0.017	1.089±0.010	1.094±0.006
	HGI	1.336±0.006	1.358±0.019	1.329±0.012	1.338±0.007
	HGBLR	1.221±0.005	1.250±0.018	1.221±0.011	1.224±0.006
Average daily gains (kg)		0.462±0.007	0.458±0.024	0.452±0.011	0.470±0.007

LSM in a column with no common superscripts differ significantly, low-case character represents significance at $p<0.05$, capital character represents significance at $p<0.01$.

locus had higher BLI than those with genotype *AA* ($p<0.05$).

The animals with genotype *AA* at *MYF5* locus were excluded when association analysis was carried out because the sample size for AA genotypes was too small.

No statistically significant differences in growth traits at *MYF5* locus were observed between the *AB* and *BB* genotypes of the Nanyang and Jiaxian breeds. However, in the Qinchuan breed animals with *BB* genotype showed lower WH and HHC when compared with *AB* genotypes ($p<0.05$).

DISCUSSION

Myostatin acts as a negative regulator of skeletal muscle growth and keeps the skeletal musculature within appropriate proportions (Lee and McPherron, 1999). Piedmontese crossbreds with an inactive *MSTN* allele have higher birth weights and yearling weights (Casas et al., 1999). Investigations of myostatin developmental expression in bovine skeletal muscles (Shibata et al., 2003) and its function in myogenesis and adipogenesis showed myostatin expression is related to animal growth (Lin et al., 2002; Joulia et al., 2003; Rebbapragada et al., 2003; Wagner et al., 2005). Mutations in *MSTN* promoter could lead to changes of the gene expression and thereby influence cattle growth and development. A T/A transversion at -371 (relative to ATG start codon) has been identified in the promoter region of porcine *MSTN* (Strail and Kopecny, 1999). A study with pigs analyzed the relationship between this mutation and growth traits, showing that individuals

Table 4. Least squares means, and standard errors for the effect of *MSTN* and *MYF5* genes on growth traits in Qinchuan and Jiaxian breeds

Breeds	Traits	Genotypes at *MSTN* gene		Genotypes at *MYF5* gene	
		AA	*AB*	*AB*	*BB*
Qinchuan	WH (cm)	93.113±0.633	89.000±2.922	94.200 [a]±0.900	92.553 [b]±0.744
(Four months)	HHC (cm)	97.266±0.648	95.833±2.995	99.096[a]±0.994	96.211[b]±0.882
	BL (cm)	93.571±0.939	95.333±4.336	94.346±1.511	93.395±1.249
	HBW (cm)	9.439±0.272	8.333±1.257	9.704±0.426	9.268±0.352
	RL (cm)	31.774±0.331	33.000±1.529	32.404±0.516	31.514±0.426
	HG (cm)	112.042±1.058	112.833±4.885	113.046±1.714	111.566±1.418
	HW (cm)	25.206±0.409	24.667±1.887	25.785±0.640	24.929±0.530
	BLI	1.005[a]±0.006	1.071[b]±0.029	1.001±0.010	1.009±0.008
	HGI	1.203±0.007	1.268±0.034	1.200±0.011	1.206±0.009
	HGBLR	1.200±0.007	1.185±0.031	0.836±0.010	0.837±0.009
Jiaxian	WH (cm)	125.463±0.501	124.200±2.263	124.740±0.662	125.813±0.630
(Twenty- four months)	HHC (cm)	124.132±0.514	122.400±2.320	123.969±0.732	124.019±0.696
	BL (cm)	154.100±0.744	151.200±3.359	152.740±1.112	155.011±1.058
	HBW (cm)	23.196±0.216	22.600±0.974	22.896±0.313	22.887±0.298
	RL (cm)	47.574±0.262	47.200±1.185	47.354±0.379	47.736±0.361
	HG (cm)	178.424±0.838	174.800±3.784	177.375±1.262	178.792±1.201
	HW (cm)	44.191±0.324	43.400±1.462	43.906±0.471	44.170±0.448
	BLI	1.229±0.005	1.219±0.022	1.225±0.007	1.233±0.007
	HGI	1.422±0.006	1.409±0.026	1.422±0.008	1.421±0.008
	HGBLR	1.161±0.005	1.157±0.024	1.163±0.008	1.157±0.007

LSM in a column with no common superscripts differ significantly, low-case character represents significance at $p<0.05$.

with *AB* genotype had a higher average daily gain than those with *AA* genotype (Jiang et al., 2002). Crisa et al. (2003) found no statistically significant differences with the mutation among genotypes of nine cattle breeds, but a significant difference between individuals bearing the combination mh/+ at the third exon and *AA* at the *Dra*I site, versus +/+ at exon 3 and *AB* or *BB* at the *Dra*I site, with an index about 30% higher in the former group. In the present study, the mutation had significant effects on WH, HG HGI and HGBLR ($p<0.05$) and the traits affected significantly were different at different growth stages, which indicated that the selected traits and the growth stages should be comprehensively considered in the breeding practice for Nanyang cattle. The mutation is located within two transcriptional factor-binding motifs and could affect the binding of these factors. The allele *B* (nucleotide A at the position) at the *Dra*I site generates a Pit1 and a WAP-US6 binding motif at -372 and at -374 respectively (Crisas et al., 2003). These results indicate a regulatory role for the promoter region in myostatin expression. In the published bovine sequence (Genbank accession number AF 348479), a C base is found at position -360 (relative to start codon), whereas Crisas et al. (2003) detected an A base at that position. There may be a polymorphism in bovine *MSTN* promoter which can be detected by PCR-RFLP with *Taq*I. This study confirmed the presence of nucleotide A at –360 (relative to start codon) after digestion of the *MSTN* promoter fragment with *Taq*I. Therefore no polymorphism was present in the PCR fragment after digestion with *Taq*I in this study and we suspect an occurrence of sequencing error in the published bovine sequence (Genbank accession number AF 348479). The result is contrary to the study by Min (2005) on goats and may contribute to the difference between species.

Knowledge of the possible mutations at the promoter region of *MSTN* gene helps elucidate the regulatory mechanisms of the gene in mammalian species. The observations have economic implications because the finding could lead to the development of selection plans that can be useful both when the double muscling trait is considered advantageous (breeding in controlled conditions), and when the trait should be avoided (range breeding). Since myostatin plays a role in breeds not carrying double muscling, the mutations occurring at the promoter region can be useful also to fine tune selection schemes aimed to obtain a desired grade of muscularity.

The *MYF5* gene has been considered to play an important role in growth and development of mammals. Li et al. (2002a, b) fine-mapped QTL for birth weight, preweaning average daily gain and average daily gain on feed on bovine chromosome 5. Three chromosomal regions (0 to 30 cM, 55 to 70 cM and 70 to 80 cM) of chromosome 5 were identified as having significant associations with growth traits. *MYF5* gene was considered to be a positional candidate gene underlying one of the chromosomal regions (0 to 30 cM) as it was mapped at the chromosomal location of 19.0 cM (Grosse et al., 1999). Extensive investigations led to the detection of several polymorphisms in *MYF5*

gene in swine and cattle. However, association analyses mostly revealed contradictory results in different lines and breeds or failed to show any relationship to production traits (Strail and Cepica, 1999; Te Pas et al., 1999; Drogemuller and Kempers, 2000; Cieslak et al., 2002; Li et al., 2002; Urbanski and Kuryl, 2004; Klosowska et al., 2004; Fausto et al., 2005). In this study, the association between the SNP of *MYF*5 and birth weight of Nanyang cattle did not reach a level of significance, which is consistent with the results of Li et al. (2004). The association between the SNP of *MYF*5 and the average daily gain of Nanyang also did not reach a significant level, whereas Li et al. (2004) and Chung et al. (2005) showed that the SNP had a significant effect on the average daily gain of cattle. In the Qinchuan population, only association between the SNP of *MYF*5 and WH and HHC reached a level of significance ($p<0.05$). In short, results of extensive investigations suggest that *MYF5* gene may be one of the causative genes that control growth traits in beef cattle or that the gene is very close to the causative genes. Being located in one of the intron regions, the SNP studied here may not be a causal mutation. Thus, one may suggest that it is linked to another mutation in the coding or regulatory regions of the gene which is a causal mutation for the growth traits. However, introns have been shown to affect transcriptional efficiency of numerous genes in a variety of organisms (Greenwood and Kelsoe, 2003; LeHir et al., 2003). An association study using more SNPs of *MYF*5, including SNPs in the promoter and extronic regions, and further functional tests of candidate genes may lead to the identification of causative mutations in the genes that control growth traits in beef cattle. These studies will be of practical importance for the improvement of Chinese native cattle and the breeding of genuine beef cattle in China.

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