Detection of SNPs in the BMP6 Gene and Their Association with Carcass and Bone Traits in Chicken

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ABSTRACT

BMP6, a member of the subfamilies of the morphogenetic proteins (BMPs), plays a crucial role in osteogenic and chondrocyte differentiation in vitro and stimulates chondrogenesis, making chondrocytes differentiate on their terminal stage. The objective of this study is to explore the relationship between polymorphism of BMP6 gene and slaughter traits in chicken respectively. We screened the exonic and intronic regions of BMP6 gene by DNA pool construction and amplified DNA fragment by PCR, and finally, we got nine SNPs. Association analysis revealed that BMP6 had no significant association among all slaughter traits in Yellow bantam chicken. However, BMP6 had a significant difference with femur weight, tibia weight, femur length ($p<0.05$), and was extremely significant with tibia length ($p<0.01$) in Avian chicken. Moreover, femur perimeter also had significant correlation with BMP6 in Avian chicken. These results provide useful information for further investigation on the function of chicken BMP6 gene.

INTODUCTION

BMP6 is a member of the subfamilies of the morphogenetic proteins (BMPs) which belongs to the transforming growth factor-β (TGF-β) superfamily of cytokine (Mehler *et al.,* 1997). In 1965, the BMPs was derived from demineralized bone extract to induce endochondral osteogenesis in vivo (Urist, 1965), and its protein-coding regions were sequenced in 1990s (Celeste *et al.,* 1990). The Vg1 gene in Xenopus is a candidate for primary axis formation (Weeks and Melton, 1987) and BMP6 was isolated with Vg1 gene by low-stringency (Lyons *et al.,* 1989). BMP6 plays crucial roles in osteogenic and chondrocyte differentiation in vitro (Sato *et al.,* 1999). Solloway *et al.* had suggested that mutations in both BMP5 and BMP6 have synergistic effects of the sternum development (Solloway *et al.*, 1998). In particular, BMP6 stimulates chondrogenesis and makes chondrocytes differentiate on their terminal stage (Grimsrud *et al.,* 1999). In recent years, more and more researches have showed that BMP6 performs a lot of functions not only on osteogenic and chondrocyte differentiation. For example, BMP6 orchestrates iron metabolism (Camaschella, 2009), and lacking of BMP6 was found to induce massive iron overload (Meynard *et al.*, 2009). Corradini *et al.* (2011) suggested that liver is the presidential source of BMP6 to regulate hepcidin and liver iron regulates the BMP6-SMAD signaling pathway with serum differently. Besides, BMP6 is also vital in reproduction. BMP6 can function as FSH stimulators for its messenger RNAs detected in pituitaries of mouse (Huang *et al.,* 2001). Sugiura *et al.* (2010) assessed BMP6 promotes normal fertility in female mice partially in a proper way by responding to LH and normal oocyte quality.

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In addition, other studies have previously reported that BMP6 gene is associated with juiciness and tenderness of the meat in pig (Fonseca *et al.,* 2003), while there is few research combing BMP6 with chicken slaughter performance so far. We compared slaughter performance between two absolute different kinds of chicken, the Avian and the Bantam yellow, for the first one is fast-growing broilers and the second is local variety in China.

MATERIALS AND METHODS

Ethics Statement

This study was performed with the approval of the Committee on Experimental Animal Management of Sichuan Agricultural University, permit number 2014-18.

Chicken populations and the collection of data

A total of 140 animals including Avian chicken (n=70) and Yellow bantam chicken (n=70) were randomly selected from commercial populations and used in the association analysis. All chickens involved in this study were raised in an experimental farm for poultry breeding at the Sichuan Agricultural University (Ya'an, China). The Avian chicken grow fast and have large bone and heavy body weight, but the Yellow bantam chicken with yellow plumage, short shanks and normal body weight. During the growth period, all birds had free access to food and water ad libitum under the same temperature and lighting conditions. The chicken were slaughtered at 70 days of age following a 12 hour fasting. Cervical dislocation was manually applied before bleeding of the neck. Blood samples were collected during bleeding and the genomic DNA was isolated by the standard phenol/chloroform method. The purity and concentration of them were assessed by a NanoVue Plus™ spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Based on the machine reading of the concentrated stocks, TE buffer was added to DNA samples extracted from blood to produce a target concentration of 100 ng/μL. The DNA samples were stored at -20°C until use.

Fifteen traits related to performance, carcass composition and bone integrity were evaluated.

At 70d of age, living weight (BW) was obtained on chickens after a 12 hour food withdrawal. After bleeding, the chicken were scalded in a hot water bath (80-90°C for 40 s) and the feathers removed mechanically, then, carcass weight (CW), eviscerated weight (EW), semi-eviscerated weight (SEW), breast muscle weight (BMW), leg muscle weight (LMW), abdominal fat weight (AW), liver weight (LW), tibia weight (TW), femur weight (FW), tibia length (TL), femur length (FL), tibia diameter (TD), femur diameter (FD), tibia perimeter (TP) and femur perimeter(FP) were obtained. All of these performance traits were determined as described in "The Poultry Production Performance Terms and Measurement Statistics Method" (NY/T823-2004).

BMP6 gene amplification and genotyping

Eight pairs of primers (Table 1) were designed to amplify 300-550 bp of exonic and intronic regions based on the chicken BMP6 gene sequence (EMBL ID: ENSGALG00000012787). Primers were synthesized by Shanghai Yingjun Biotechnology Co. Ltd. (Shanghai, China). Sequences were obtained from Avian and Yellow bantam chicken DNA pool (30 random chicken DNA samples in the each DNA pool). To amplify DNA fragment of BMP6 gene, a PCR reaction was performed in 25 μL containing 2 μL of pooled DNA, 1.25 μL (10 pmol/μL) of each primer, 12.5 μL 2×Master mix (including Mg^{2+}, dNTPs, *Taq* DNA polymerase; Beijing TIAN WEI Biology Technique Corporation, Beijing, China), and finally adjusting the volume to 25 μL by adding ultrapure water. The PCR reactions were carried out in EasyCycler 96 PCR detection system (Analytik Jena, Germany). We used a PCR protocol under the following condition: denaturing at 95°C for 5 min;

Table 1 – BMP6 primer sequence

Name	Sequence (5'- 3')	Production (bp)	Anneal temperature (°C)
1	F: CCTTGAGAAGAATGCCACGTT R: CCTTGGTATGTGCCAGGAAAT	444	57.8
2	F: GCATAAGATTTCCTGGCACATAC R: TCTACTATGACCATTTCCCACAAG	471	57.8
3	F: GTGGAAGAACTCTGGATACAAACA R: GCTCATTCCCAACATTACAACTG	410	55.4
4	F: TCTCGGATGGACAGGTTGC R: TTCCTTCTCCCGTTCTATTGC	535	56.6
5	F: CTGTCCGTCAGCACTCTTCTCT R: TTAAAGCATAATGGCAGCCTC	291	57.8
6	F: GCACCATTCTTTGGACACGA R: GGTTTGGGAACGTAATCAGGA	443	56.6
7	F: ATGAACGCAACCAACCACG R: TGCCTTGGATTATGATACGGA	2017	56.6
8	F: GGTTTGCTGCTGTCATTGTACT R: ATAGTGTATGTGTCAGGTGGATGTT	495	57.8

followed by 35 cycles of denaturing at 95°C for 40 s, annealing at 55°C (or other apt annealing temperature as shown in Table 1) for 35 s, and extension at 72°C for 45 s. The final extension was performed at 72°C for 7 min. PCR products were purified with a gel extraction kit (Takara, Dalian, China) and sequenced on an ABI 377 DNA sequence (Shanghai Sangon Biological Engineering Technology, Shanghai, China). Sequences were analyzed with the DNASTAR software and the CodonCode Aligner software (http: //www. codoncode.com/aligner).

Based on the sequencing of the two DNA pools, polymorphisms were identified with four of the primer pairs. Genotyping was performed using DNA samples extracted from blood samples collected from the 140 chickens. To analyze the mutations, PCR was performed as described above. Amplified products were electrophoresed and purified with a gel extraction kit (Takara, Dalian, China) and sequenced by Shanghai Sangon Biology Technique Corporation.

Data analysis

Genotypic and allelic frequencies were calculated by counting the genotypes and alleles for each SNP

and Hardy-Weinberg equilibrium was established with chi-square test at 5% significance level. The linkage disequilibrium (LD) structure as measured by D' and r^2 were performed with the Haploview software (Version 3.32) (Barrett *et al.*, 2005).

The general linear model (GLM) procedure of JMP 10 was used to test associations between the genotyped markers and carcass traits. The model is as follows:

$$Y_{ijk} = \mu + S_i + G_j + B_k + G_j \times S_i \times B_k + e_{ijk}$$

where Y is the trait measured on chickens, μ is the population mean, S_i is the fixed effect of sex, G_j is the fixed effect of genotype, B_k is the fixed effect of breed, $G_j \times S_i \times B_k$ is the interaction among genotype, sex and breed, and e is the random error. The values were presented as least square means±se. Statistical significance was evaluated using Duncan's test. Differences were considered significant at $p<0.05$.

Haplotypes were constructed using the Haploview program (version 3.32, http://www.broad.mit.edu/mpg/haploview/). Haplotypes were analyzed using the model applied for the single marker association test with consideration for animals having 0, 1, or 2 copies of the haplotype in question. The PROC REG

Table 2 – Genotypic and allele frequencies of four single nucleotide polymorphisms of BMP6 gene among Avian populations.

SNP	Genotype	Number	Genotype Frequency	Allele Frequency	P	χ^2
64487388A>G	AA (A)	37	0.544	0.757	0.186	1.748
	AG	29	0.426	-		
	GG (G)	2	0.03	0.243		
64475440C>T	CC (C)	16	0.235	0.471	0.647	0.209
	CT	32	0.471	-		
	TT (T)	20	0.294	0.529		
64474334G>C	GG (G)	5	0.074	0.463	p<0.01	21.884
	GC	53	0.779	-		
	CC (C)	10	0.147	0.537		
64487436A>C	AA (A)	39	0.574	0.773	0.29	1.116
	AC	27	0.397	-		
	CC (C)	2	0.029	0.227		
64487410G>A	GG (G)	36	0.529	0.765	0.01	6.438
	GA	32	0.471	-		
	AA (A)	0	0	0.235		
64474352T>C	TT (T)	44	0.647	0.816	0.294	1.1
	TC	23	0.338	-		
	CC (C)	1	0.015	0.184		
64474300C>T	CC (C)	37	0.544	0.75	0.418	0.654
	CT	28	0.412	-		
	TT (T)	3	0.044	0.25		
64474290C>G	CC (C)	37	0.544	0.757	0.186	1.748
	CG	29	0.426	-		
	GG (G)	2	0.03	0.243		
64463906T>C	TT (T)	4	0.059	0.309	0.158	1.994
	TC	34	0.5	-		
	CC (C)	30	0.441	0.691		

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Table 3 – Genotypic and allele frequencies of four single nucleotide polymorphisms of BMP6 gene among Yellow bantam populations.

SNP	Frequency				P	χ^2
	Genotype	Number	Genotype	Allele		
64487388A>G	AA(A)	47	0.671	0.807	0.284	1.149
	AG	19	0.271	-		
	GG(G)	4	0.058	0.193		
64475440C>T	CC(C)	38	0.543	0.736	0.945	0.005
	CT	27	0.386	-		
	TT(T)	5	0.071	0.264		
64474334G>C	GG(G)	14	0.2	0.429	0.577	0.311
	GC	32	0.457			
	CC(C)	24	0.343	0.571		
64487436A>C	AA(A)	47	0.671	0.814	0.643	0.214
	AC	20	0.286			
	CC(C)	3	0.043	0.186		
64487410G>A	GG(G)	36	0.514	0.729	0.484	0.489
	GA	30	0.429			
	AA(A)	4	0.057	0.271		
64474352T>C	TT(T)	48	0.686	0.8	0.017	5.714
	TC	16	0.229			
	CC(C)	6	0.085	0.2		
64474300C>T	CC(C)	44	0.629	0.765	0.039	4.259
	CT	19	0.271			
	TT(T)	7	0.1	0.235		
64474290C>G	CC(C)	40	0.571	0.728	0.086	2.952
	CG	22	0.314			
	GG(G)	8	0.115	0.272		
64463906T>C	TT(T)	21	0.3	0.564	0.531	0.392
	TC	37	0.529			
	CC(C)	12	0.171	0.436		

procedure of SAS (version 6.12, SAS Institute Inc.) was used to perform the analysis. Significant associations were declared at $p < 0.05$.

RESULTS

Identification of SNPs in the chicken BMP6 gene

We sequenced the individuals of the random population genotypes and found nine mutations, including a A/G mutation (g.64487388A>G), a C/T mutation (g.64475440C>T), a G/C mutation (g.64474334G>C), a A/C mutation (g. 64487436A>C), a G/A mutation (g.64487410G>A), a T/C mutation (g.64474352T>C), a C/T mutation (g.64474300C>T), a C/G mutation (g.64474290C>G) and a T/C mutation (g.64463906 T>C). Table 2 and Table 3 show the Genotypic and allelic frequencies of nine single

nucleotide polymorphisms of *BMP6* gene among the two different strains of populations.

### Allele and Genotype Frequency of the Mutated sites

The Chi-squared test was conducted to compare the allele and genotypes frequency in the BMP6 gene of all 7 SNPs between Avian and Yellow bantam populations and the results were shown in Table 2 and Table 3. The data indicates that the homozygous genotype were dominant compared with heterozygotes genotype in both Avian and Yellow bantam populations of SNP1, SNP4, SNP5, SNP6, SNP7, SNP8, therefore, the homozygous GG genotype were dominant compared with homozygous AA genotype in SNP1, SNP5, and the homozygous AA genotype predominated in SNP4, the homozygous TT genotype predominated in SNP6, the homozygous CC genotype predominated in SNP7

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and SNP8 in both populations. For SNP3, SNP9, the heterozygotes were advantageous compared with homozygous. As for SNP2, CT genotypes predominated in the Avian population and T was the advantageous allele while in the Yellow bantam population, C was the advantageous allele and CC were dominant.

The Hardy-Weinberg Equilibrium

Figure 1 indicates that the degree of the linkage disequilibrium (LD) revealed the correlation between polymorphic variants at different positions of BMP6 gene. Obviously, the SNPs of block 1 (SNP3, SNP6, SNP7 and SNP8) and block 2 (SNP1, SNP4 and SNP5) are of high D' respectively according to 2 blocks in dark red in the D'. Nevertheless, SNP2 and SNP9 were in equilibrium and independent in both blocks. Haplotype analysis showed (Table 4) that the haplotype groups CCGT (χ^2=1.519, p=0.2178), CCCT (χ^2=4.661, p=0.0309), GTCC (χ^2=0.445, p=0.5047), GTCT (χ^2=0.108, p=0.7426), GCCT (χ^2=0.035, p=0.8521) in block1 and groups AGA (χ^2=1.987, p=0.1586), AAA (χ^2=0.738, p=0.3903), GGC (χ^2=0.489, p=0.4842), GGA (χ^2=0.045, p=0.8317), AGC (χ^2=0.023, p=0.8807). The Hardy-Weinberg equilibrium tests of the 9 SNPs were shown in Table 4. The heterozygosity of all SNPs were observed as expected and most of SNPs fit the assumption of the Hardy-Weinberg equilibrium except SNP2 (g.64475440C>T) and SNP9 (g.64463906 T>C)(D'<0.75). While the minor allele frequencies (MAF) of all the mutations were more than 0.01. (SNP1: 0.217, SNP2: 0.395, SNP3: 0.438, SNP4: 0.207, SNP5: 0.268, SNP6: 0.192, SNP7: 0.236, SNP8: 0.261, SNP9: 0.435).
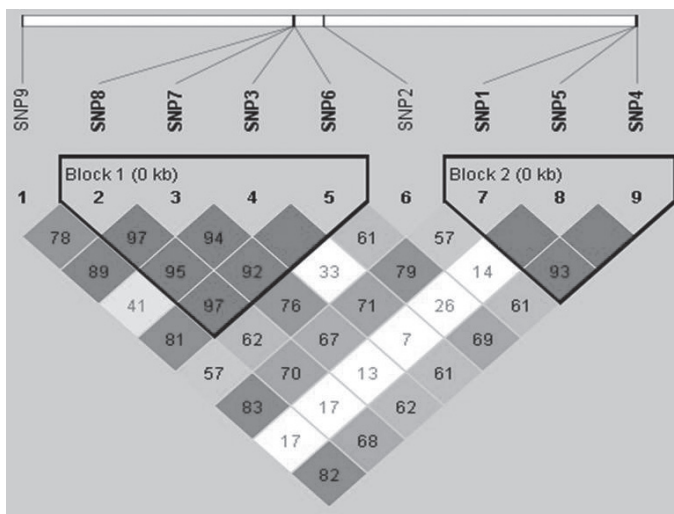
Figure 1 – LD value within each diamond represents the correlation between pairs of SNPs (measured as D') in the introns and exons of BMP6 gene. The diamond without a number means complete LD (D'=1). Darker red of the diamonds indicates higher D', while white indicates lower D'.

Table 4 – The haplotype analysis of 7 BMP6 SNPs.

	Haplotypes groups	Frequency	χ^2	p-value
	CCGT	0.43	1.519	0.2178
	CCCT	0.302	4.661	0.0309
block 1	GTCC	0.181	0.445	0.5047
	GTCT	0.051	0.108	0.7426
	GCCT	0.017	0.035	0.8521
	AGA	0.503	1.987	0.1586
	AAA	0.268	0.738	0.3903
block 2	GGC	0.195	0.489	0.4842
	GGA	0.022	0.045	0.8317
	AGC	0.011	0.023	0.8807

Correlation analysis of the SNPs in BMP6 gene and carcass traits

According to the results of the least-squares analysis (Table 5), we could find that all the SNPs had no significant correlation with all carcass traits in both populations of chicken, which means BMP6 gene affected little in carcass traits in Avian and Yellow bantam chicken. To find if BMP6 gene was expressed in the tissues of carcass traits or not, further study is required.

Correlation analysis of the SNPs in BMP6 gene and skeleton traits

In Table 6, the results indicated that SNP5 showed significant difference among FW, TW, FL (p<0.05) and extremely significant difference with TL (p<0.01) in the Avian population. In addition, SNP1, SNP4 both had significant correlation with FP (p<0.01) in the Avian population. However, there were no significant differences between SNPs and skeleton traits in the Yellow bantam population.

Correlation analysis of the BMP6 haplotypes and carcass traits

The comparative results of the least squares mean of each character among BMP6 haplotypes are shown in Table 7 and Table 8. The BMP6 haplotypes had significant differences in both Avian and Yellow bantam population respectively. In the Yellow bantam populations, we found CCGT, CCCT, GTCC, GTCT, GCCT, AAA, AGA, GGC, GGA haplotypes individuals in the population. CCCT haplotype individuals were significantly different in CCGT haplotype individuals in AFW (p<0.05), GTCT haplotype individuals had significant differences in CCGT haplotype individuals in LW (p<0.05). There were significant differences between GGA haplotype individuals and GGC haplotype individuals in FP (p<0.05). In the Avian populations,

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Table 5 – Association of the single locus of *BMP6* gene and carcass traits

Sites	Strians	Genotypes	LW	EW	SEW	BMW	LMW	AFW	LW
						Carcass traits (g)			
SNP1(64487388A>G)	Avian	AA	2957.05±68.41	1999.36±52.10	2400.37±589.90	280.07±10.27	259.24±7.44	22.10±2.52	54.03±1.75
		AG	3023.52±79.34	2008.62±60.38	3514.31±684.10	297.00±11.91	261.42±8.62	19.38±2.92	52.96±2.03
		GG	2750.00±302.11	1850.00±229.92	2267.50±2602.96	296.55±45.35	219.10±32.84	9.25±11.14	45.95±7.74
	Yellow bantam	AA	1629.89±57.10	960.67±34.91	1243±48.69	80.32±3.03	110.84±5.76	25.00±2.54	33.63±1.46
		AG	1663.16±87.87	991.53±53.73	1270±74.94	83.36±4.67	115.09±8.86	31.11±3.91	34.79±2.24
		GG	1587.50±191.51	953.75±117.11	1274±163.32	89.06±10.17	110.91±13.92	28.61±8.53	30.50±4.88
SNP2(64474334G>C)	Avian	CC	3097.50±133.31	2070.00±101.71	2523.50±1175.37	308.53±20.24	258.24±14.60	28.74±4.92	55.03±3.45
		CG	2983.93±57.37	2003.24±43.77	2997.22±505.80	285.35±8.71	262.17±6.28	19.02±2.12	53.57±1.49
		GG	2733.33±172.11	1841.67±131.31	2163.25±1517.39	272.41±26.13	231.73±18.85	21.31±6.35	48.54±4.46
	Yellow bantam	CC	1720.83±77.02	1001.25±47.49	1308.54±65.95	84.73±4.15	118.51±7.82	30.59±3.48	35.32±1.98
		CG	1570.16±67.78	936.74±41.79	1200.48±58.03	79.57±3.65	106.77±6.88	25.06±3.06	32.07±1.74
		GG	1636.54±104.65	985.77±64.53	1263.46±89.60	81.08±5.64	112.60±10.62	24.60±4.73	34.96±2.69
SNP3(64487436A>C)	Avian	AA	2265.12±84.28	1458.49±63.34	1799.59±194.33	176.02±12.34	182.65±9.14	23.86±1.80	43.31±1.53
		AC	2449.62±114.01	1577.11±85.68	2605.11±398.14	206.63±16.56	198.66±12.37	23.35±2.44	45.23±2.07
		CC	1965.00±349.55	1251.00±262.69	1581.00±1220.67	166.70±50.76	140.67±37.94	24.78±7.48	36.07±6.35
	Yellow bantam	AA	1621.67±56.58	957.89±34.58	1240.11±48.41	80.13±3.03	110.95±5.69	25.61±2.55	33.61±1.45
		AC	1697.5±84.87	1011.2±51.86	1293.5±129.35	85.40±4.55	118.02±8.54	28.65±3.83	34.76±2.18
		CC	1697.5±219.13	851.67±133.91	1123.33±112.33	80.13±11.74	88.38±22.05	35.14±9.89	29.49±5.63
SNP4(64487410G>A)	Avian	AA	1568.75±388.95	896.25±291.03	1900.00±1373.71	79.83±56.41	105.37±42.20	16.60±8.32	16.60±7.10
		AG	2373.86±95.75	1538.18±71.64	1905.45±337.19	195.35±13.89	192.12±10.39	25.20±2.05	25.20±1.75
		GG	2306.99±94.33	1480.94±70.59	2273.38±333.17	183.39±13.68	185.98±10.24	22.71±2.02	22.71±1.72
	Yellow bantam	AA	1683.33±220.62	958.33±135.47	1283.33±188.68	84.97±11.81	116.83±22.32	12.14±9.78	36.92±5.65
		AG	1599.14±70.96	971.03±43.57	1248.79±60.68	80.77±3.80	112.17±7.18	28.70±3.15	33.47±1.82
		GG	1661.81±63.69	968.03±39.11	1249.44±54.47	82.14±3.41	112.17±6.44	26.72±2.82	33.74±1.63
SNP5(64474352T>C)	Avian	CC	1841.67±318.90	1189.17±239.60	1490.83±1124.09	124.41±46.10	138.22±34.64	20.29±6.81	37.41±5.81
		CT	2419.23±125.08	1570.03±93.98	1948.60±440.91	209.79±18.08	196.86±13.59	25.80±2.67	43.31±2.28
		TT	2304.91±81.00	1477.88±60.86	2152.36±285.52	180.16±11.71	185.39±8.80	23.07±1.73	44.27±1.48
	Yellow bantam	CC	1675.00±170.97	1063.00±103.67	1350.00±144.90	90.27±9.08	119.62±17.26	24.07±7.53	36.25±4.35
		CT	1582.80±95.57	919.10±58.96	1186.60±81.00	79.33±5.08	108.59±9.65	33.85±4.21	31.71±2.43
		TT	1650.00±55.76	975.80±33.81	1261.90±47.26	81.56±2.96	112.40±5.63	24.87±2.45	34.20±1.42
SNP6(64474300C>T)	Avian	CC	2268.53±87.18	1459.98±65.45	2180.43±307.79	175.64±12.55	182.90±9.49	23.49±1.86	43.92±1.60
		CT	2464.89±113.74	1593.32±85.39	1974.26±401.56	214.41±16.37	199.44±12.38	25.21±2.43	44.04±2.08
		TT	2038.64±235.11	1284.09±176.51	1625.91±830.05	141.35±33.85	158.39±25.59	19.00±5.02	40.67±4.31
	Yellow bantam	CC	1629.27±59.10	969.22±35.96	1247.68±50.10	80.63±3.15	110.29±5.97	26.42±2.65	33.51±1.48
		CT	1584.21±86.82	922.68±52.82	1191.84±73.58	80.05±4.63	108.98±8.76	30.90±3.89	31.59±2.17
		TT	1793.75±133.80	1076.88±81.40	1405.63±113.40	90.94±7.14	128.20±13.51	20.06±6.00	40.29±3.35
SNP7(64474290C>G)	Avian	CC	2287.43±89.03	1475.33±66.77	2211.49±313.55	179.16±12.84	184.97±9.68	23.03±1.90	44.31±1.63
		CG	2426.47±109.39	1565.96±82.04	1940.98±385.27	207.28±15.78	195.74±11.89	25.57±2.33	43.38±2.00
		GG	1987.50±247.04	1234.50±185.28	1583.50±870.06	131.63±35.63	152.26±26.86	19.58±5.26	40.70±4.52
	Yellow bantam	CC	1617.11±61.62	961.58±37.64	1236.97±52.26	80.26±3.29	108.76±6.18	25.72±2.74	33.47±1.55
		CG	1617.05±80.98	947.91±49.47	1220.90±68.68	81.54±4.33	111.14±8.12	31.35±3.61	32.09±2.03
		GG	1778.13±134.30	1061.25±82.03	1397.50±113.90	88.83±7.18	130.02±13.46	20.48±5.98	39.80±3.37

Table 6 – Association of the single locus of *BMP6* gene and bone traits

Sites	Breeds	Genotypes	Skeleton traits							
			FW(g)	TW(g)	FD(mm)	TD(mm)	FL(cm)	TL(cm)	FP(cm)	TP(cm)
SNP1(64487388A>G)	Avian	AA	20.20±0.70	30.16±1.25	10.09±0.19	9.20±0.32	9.09±0.10	12.23±0.12	4.84±0.07[a]	4.58±0.11
		AG	20.78±0.81	30.92±1.45	10.07±0.22	9.47±0.37	9.22±0.12	12.38±0.13	4.87±0.08[a]	4.58±0.12
		GG	17.70±3.08	29.55±5.54	10.03±0.85	8.58±1.42	9.24±0.45	12.15±0.51	4.00±0.43[b]	3.95±0.47
	Yellow bantam	AA	13.00±0.68	17.57±1.11	9.00±0.26	7.47±0.19	7.93±0.14	10.92±0.23	4.53±0.05	4.08±0.06
		AG	12.13±1.04	16.35±1.70	8.79±0.39	7.50±0.30	7.89±0.22	10.96±0.36	4.47±0.08	4.05±0.10
		GG	14.77±2.27	15.81±3.72	8.81±0.86	7.75±0.64	7.93±0.47	10.61±0.78	4.65±0.17	4.23±0.22
SNP2(64474334G>C)	Avian	CC	20.04±1.38	29.37±2.46	10.01±0.38	9.13±0.63	9.28±0.20	12.32±0.22	4.53±0.14	4.32±0.21
		CG	20.54±1.60	30.97±1.06	10.12±0.16	9.43±0.27	9.16±0.09	12.34±0.10	4.78±0.06	4.58±0.10
		GG	19.36±1.79	27.69±3.17	9.817±0.49	8.28±0.81	8.81±0.26	11.79±0.29	4.95±0.19	4.83±0.27
	Yellow bantam	CC	13.63±0.92	17.89±1.52	9.12±0.35	7.82±0.26	8.03±0.19	11.17±0.31	4.58±0.07	4.18±0.09
		CG	12.17±0.81	16.74±1.34	8.78±0.31	7.29±0.23	7.83±0.17	10.66±0.28	4.50±0.06	4.05±0.08
		GG	13.08±1.25	16.64±2.07	8.78±0.47	7.37±0.35	7.93±0.26	11.07±0.43	4.47±0.09	3.96±0.12
SNP3(64487436A>C)	Avian	AA	20.25±0.68	30.30±1.22	10.06±0.19	9.15±0.31	9.11±0.10	12.25±0.11	4.73±0.07[a]	4.55±0.10
		AC	20.76±0.84	30.76±1.51	10.11±0.23	9.56±0.39	9.20±0.12	12.36±0.14	4.83±0.09[a]	4.62±0.13
		CC	17.70±3.08	29.55±5.54	10.03±0.85	8.58±1.42	9.24±0.45	12.15±0.51	4.00±0.43[b]	3.95±0.47
	Yellow bantam	AA	12.63±0.68	17.01±1.11	8.90±0.26	7.43±0.19	7.92±0.14	10.95±0.23	4.54±0.05	4.08±0.06
		AC	13.34±1.02	17.86±1.66	8.93±0.38	7.67±0.29	7.93±0.21	10.90±0.35	4.49±0.08	4.08±0.10
		CC	13.08±2.63	14.03±4.29	8.67±0.99	7.30±0.74	7.83±0.54	10.60±0.90	4.50±0.20	4.00±0.25
SNP4(64487410G>A)	Avian	AA	9.85±4.18[b]	7.21±7.16[b]	8.00±1.16	5.25±1.96	7.13±0.59[b]	9.38±0.63[B]	4.70±0.49	4.00±0.64
		AG	20.14±0.69[a]	29.50±1.18[a]	10.00±0.19	9.21±0.32	9.15±0.10[b]	12.26±0.10[B]	4.72±0.08	4.42±0.11
		GG	20.96±0.74[a]	29.50±1.19[a]	10.28±0.20	9.51±0.35	9.21±0.10[a]	12.42±0.11[A]	4.80±0.08	4.75±0.11
	Yellow bantam	AA	14.25±2.63	18.51±4.31	9.38±0.97	7.93±0.74	8.26±0.54	11.17±0.90	4.73±0.19	4.10±0.25
		AG	12.81±0.85	16.97±1.39	9.21±0.31	7.50±0.24	7.95±0.17	11.03±0.29	4.50±0.06	4.06±0.08
		GG	12.78±0.76	17.14±1.24	8.61±0.28	7.45±0.21	7.87±0.16	10.81±0.26	4.52±0.06	4.09±0.07
SNP5(64474352T>C)	Avian	CC	18.90±4.39	35.00±7.82	10.25±1.19	9.25±2.01	9.35±0.63	12.46±0.72	4.10±0.46	3.80±0.66
		CT	20.48±0.91	30.67±1.63	10.17±0.25	9.55±0.42	9.27±0.13	12.39±0.15	4.78±0.10	4.53±0.14
		TT	20.35±0.65	30.25±1.15	10.03±0.18	9.16±0.30	9.09±0.09	12.24±0.11	4.76±0.09	4.59±0.10
	Yellow bantam	CC	15.15±2.00	19.22±3.28	9.90±0.75	7.95±0.57	8.33±0.42	11.54±0.68	4.66±0.15	4.18±0.19
		CT	11.44±1.12	14.74±1.83	8.48±0.42	7.40±0.32	7.73±0.23	10.48±0.38	4.47±0.08	4.12±0.11
		TT	13.10±0.65	17.72±1.07	8.93±0.25	7.48±0.19	7.94±0.14	11.00±0.22	4.53±0.05	4.05±0.06
SNP6(64474300C>T)	Avian	CC	20.55±0.69	30.55±1.24	9.98±0.19	9.17±0.32	9.05±0.10	12.18±0.11	4.78±0.07	4.59±0.11
		CT	20.50±0.82	30.90±1.46	10.29±0.22	9.48±0.38	9.33±0.12	12.48±0.13	4.75±0.09	4.54±0.13
		TT	16.80±2.50	25.10±4.78	9.32±0.68	0.90±1.16	8.78±0.36	11.99±0.41	4.57±0.27	4.30±0.38
	Yellow bantam	CC	12.92±0.70	17.46±1.14	8.90±0.26	7.41±0.02	7.85±0.14	10.88±0.24	4.50±0.05	4.03±0.07
		CT	11.67±1.02	14.97±1.67	8.39±0.38	7.33±0.03	7.84±0.21	10.58±0.35	4.48±0.08	4.09±0.10
		TT	15.38±1.57	20.54±2.57	10.01±0.58	8.28±0.04	8.47±0.32	11.88±0.53	4.70±0.12	4.26±0.15
SNP7(64474290C>G)	Avian	CC	20.55±0.70	30.55±1.25	9.98±0.19	9.17±0.32	9.05±0.10	9.05±0.11	4.78±0.08	4.59±0.11
		CG	20.31±0.81	30.52±1.45	10.22±0.22	9.42±0.37	9.31±0.12	9.31±0.13	4.75±0.09	4.53±0.12
		GG	17.75±3.09	27.65±5.53	9.75±0.84	9.63±1.43	8.84±0.44	8.84±0.50	4.55±0.33	4.30±0.47
	Yellow bantam	CC	12.68±0.73	17.17±1.20	9.00±0.27	7.40±0.20	7.79±0.15	10.82±0.25	4.49±0.05	4.02±0.07
		CG	12.40±0.96	16.03±1.57	8.53±0.36	7.47±0.27	7.95±0.20	10.76±0.32	4.50±0.07	4.12±0.09
		GG	14.96±1.59	19.93±2.61	9.91±0.59	0.80±0.45	8.45±0.32	11.81±0.54	4.71±0.12	4.26±0.15

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Table 7 – Association of the haplotypes of *BMP6* gene with bone traits and carcass traits in the Yellow bantam populations

Traits	Block1					Block2			
	CCGT	CCCT	GTCC	GTCT	GCCT	AAA	AGA	GGC	GGA
LW (g)	1633.44±135.33	1703.65±149.60	1740.76±208.85	2063.57±301.86	1883.65±407.22	1711.53±243.98	1771.36±107.29	1610.80±246.01	2126.36±407.24
EW(g)	998.23±83.32	992.33±92.11	1123.60±128.58	1135.49±185.85	1070.33±250.72	978.35±150.22	1023.66±66.05	922.31±151.46	1331.66±250.73
SEW(g)	1270.22±115.61	1283.62±127.81	1420.92±178.42	1590.46±257.88	1427.29±347.90	1304.87±208.44	1356.21±91.66	1213.05±210.17	1719.88±347.91
BMW(g)	89.48±7.24	90.06±8.00	100.05±11.17	92.97±16.14	98.20±21.78	88.48±13.05	84.55±5.74	83.65±13.16	119.92±21.78
LMW(g)	120.95±13.38	119.70±14.80	134.57±20.65	162.60±29.85	157.52±40.27	121.07±24.13	127.75±10.61	109.60±24.33	197.86±40.27
AFW(g)	15.90±5.79[b]	27.96±6.40[a]	13.99±8.94[ab]	13.92±12.92[ab]	-0.15±17.43[ab]	11.22±10.44	23.78±4.59	26.89±10.53	-4.59±17.43
LW(g)	31.48±3.40[b]	31.26±3.76[ab]	35.70±5.25[ab]	46.87±7.59[a]	39.51±10.24[ab]	37.27±6.14	38.75±2.70	32.61±6.19	39.22±10.24
FW(g)	14.26±1.61	14.33±1.77	17.19±2.48	16.66±3.59	15.06±4.84	14.69±2.90	13.68±1.27	12.63±2.92	21.00±4.84
TW(g)	16.81±2.67	16.44±2.95	20.02±4.11	22.85±5.95	17.71±8.02	18.46±4.81	18.90±2.11	14.22±4.85	23.47±8.02
FD(mm)	0.88±0.06	0.87±0.07	1.00±0.09	1.07±0.14	0.85±50.18	0.93±0.11	0.93±0.05	0.89±0.11	0.99±0.18
TD(mm)	0.77±0.05	0.81±0.05	0.84±0.07	0.91±0.10	0.71±0.14	0.78±0.08	0.76±0.04	0.78±0.08	0.91±0.14
FL(cm)	1044.25±2742.68	-623.38±3031.96	-384.58±4232.59	44.15±6117.56	-622.09±8253.07	-810.96±4944.68	522.84±2174.30	-668.47±4985.77	523.27±8253.35
TL(cm)	10.59±0.55	10.65±0.61	11.42±0.85	12.18±1.23	11.99±1.66	11.41±1.00	11.41±1.00	11.11±1.00	11.37±1.66
FP(cm)	4.63±0.12	4.61±0.13	4.84±0.18	4.98±0.26	4.74±0.35	4.75±0.21[a]	4.62±0.09[ab]	4.43±0.21[b]	5.25±0.35[a]
TP(cm)	4.18±0.15	4.25±0.17	4.35±0.23	4.71±0.34	4.35±0.45	4.11±0.27	4.21±0.12	4.13±0.27	5.02±0.45

Note: The different lowercase letters on the same line indicate significant difference (*p*< 0.05).

Table 8 – Association of the haplotypes of *BMP6* gene with bone traits and carcass traits in the Avian populations

Traits	Block1				Block2		
	CCGT	CCCT	GTCC	GTCT	AAA	AGA	GGC
LW (g)	2354.36±176.65	2413.51±217.95	2204.36±465.61	2316.29±413.79	1192.77±408.50	2980.84±166.39	2792.77±311.90
EW(g)	1538.67±136.65	1553.79±168.60	1478.66±360.17	1547.68±320.08	701.04±315.99[B]	2017.02±128.71[A]	1871.04±241.26[A]
SEW(g)	2081.75±1812.38	1579.83±2236.02	1936.75±4776.90	1546.49±4245.28	614.45±4191.02[B]	2699.71±1707.08[A]	2044.45±3199.90[A]
BMW(g)	217.48±28.23	227.28±34.83	214.58±74.41	238.07±66.13	71.30±65.28[B]	296.89±26.59[A]	304.87±49.84[A]
LMW(g)	180.79±19.49	175.23±175.23	204.99±51.37	167.61±45.66	72.35±45.07[B]	265.75±18.36[A]	208.36±34.41[A]
AFW(g)	22.17±7.49	31.39±9.24	6.47±19.74	33.33±17.54	31.14±17.32	20.61±7.05	18.27±13.22
LW(g)	42.12±4.89	42.80±6.03	36.62±12.88	33.19±11.45	19.90±11.30[b]	50.89±4.60[a]	45.26±8.63[ab]
FW(g)	15.71±1.99	15.50±2.45	18.11±5.24	11.53±4.66	9.35±4.60[b]	20.29±1.87[a]	16.00±3.51[ab]
TW(g)	20.94±3.39	18.48±4.18	31.84±8.93	9.74±7.94	6.52±7.83[b]	30.81±3.19[a]	23.41±5.98[ab]
FD(mm)	0.93±0.06	0.91±0.07	0.98±0.15	0.84±0.13	0.78±0.13	1.00±0.05	0.96±0.10
TD(mm)	0.75±0.09	0.70±0.12	0.89±0.25	0.81±0.22	0.56±0.22[b]	0.98±0.09[a]	0.82±0.17[ab]
FL(cm)	8.47±0.28	8.73±0.34	8.70±0.73	7.64±0.65	7.64±0.65[b]	9.08±0.26[a]	9.04±0.49[a]
TL(cm)	11.19±0.30	11.28±0.37	11.80±0.80	10.39±0.71	9.35±0.70[B]	12.32±0.29[A]	11.82±0.54[A]
FP(cm)	4.73±0.22	4.51±0.27	4.13±0.59	4.93±0.52	4.54±0.51	4.64±0.21	4.54±0.39
TP(cm)	4.24±0.32	4.00±0.39	3.94±0.83	4.42±0.74	3.91±0.73	4.53±0.30	4.01±0.56

Note: Different capital letters on the same line indicate extreme significant differences (*p*< 0.01); different lowercase letters indicate significant difference (*p*< 0.05).

haplotypes in block 1 (CCGT, CCCT, GTCC, GTCT) had no significant differences among characters while block 2 (AAA, AGA, GGC) were significantly correlated among most of the characteristics. There into, AAA haplotype individuals were significantly correlated in AGA and GGC haplotype individuals among EW, SEW, BMW, LMW, TL ($p<0.01$) respectively. AAA haplotype individuals were also significantly different with AGA haplotype individuals among LW, FW, TW, TD respectively ($p<0.05$). AGA and GGC haplotype individuals had significant differences with AAA haplotype individuals in FL ($p<0.05$).

DISCUSSION

Multiple genes have controlled carcass traits and skeleton traits separately (Fontanesi *et al.,* 2008; Bolormaa *et al.,* 2011). Analyzing the association between candidate genes and productive traits is an advisable way to understand whether specific genes are correlated with specific traits in economic animals and we can take advantage of DNA tests in animal breeding as selection tools (De Vries, *et al.*, 1998).

Although several studies indicated that the SNPs of the BMP6 gene is involved in peripheral bone mineral density (Choi *et al.,* 2006), avascular necrosis (Ulug *et al.,* 2009), sickle cell osteonecrosis of human (Baldwin *et al.*, 2005). At present, studies of BMP6 showed that it is more focused on human disease such as hepatic fibrosis, iron overload disease (Kleven *et al.,* 2016), prostate cancer-associated disease (Turner and Edwards, 2016) and so on. Nevertheless, BMP6 was chose to be a candidate of meat quality (Lee *et al.,* 2014).

In this study, the different variant PCR products were sequenced. Due to the rate of recombination, selection and foreign blood imported in the chicken population, the alleles and genotypes of loci 64475440C>T and loci 64463906T>C were unsuitable and were removed from statistic analysis because the Hardy-Weinberg equilibrium is impacted by many factors such as the rate of mutation, the rate of recombination, selection, genetic drift, the system of mating, population structure, and genetic linkage (Liu *et al.*, 2015).

Then we explored the correlation between SNPs and carcass, skeletal traits. In carcass traits, there were no significant differences with any carcass traits among SNPs. A previous report indicated that mice fed with high-fat diet caused reduction of BMP6 gene expression in visceral adipose tissue (Gotarod *et al.*, 2013), whereas there were no reports showing detailed mechanism of BMP6 gene in abdominal fat and it needs further research. In contrast to other BMPs

(BMP2, BMP4, BMP9), BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism (Andriopoulos *et al.*, 2009). Lacking of the BMP6 induces massive iron overload in the liver of BMP6-deficient mice (Meynard *et al.*, 2009). However, no researches showed if BMP6 is related to liver weight directly or not. As for the skeletal traits, Kugimiya *et al.*, initially investigated the size of the growth plate of proximal tibias in the $BMP2^{+/-}BMP6^{-/-}$ compound-deficient mouse which were significantly smaller than the wild types (Kugimiya *et al.*, 2005), and our studies that SNP3 in Avian was significant associated with FW, TW, FL ($p<0.05$), and TL($p<0.01$) were consistent with it. Recently, a research disclosed that exogenous heparin reduced the BMP6 osteogenic activity by using μCT analysis of femur in the mice with osteoporotic (Brkljacic *et al.*, 2013). Both SNP1 and SNP3 had significant correlation with FP ($p<0.05$). While there is no other research focused on the association between the skeletal traits and BMP6 in economic animals. Our experiment dates indicated that it seems to be no relevance between SNPs between skeleton traits in the Bantam yellow. The reasons for this phenomenon and expression pattern in these two different kinds of strains are still waiting for for discovery. All this adds up to the result that the single locus of SNPs of BMP6 has no direct correlation to carcass traits in these two chicken populations, but was relevant to skeleton traits in the Avian population.

A recent research about it has performed haplotype analysis of BMP6 and then they found eight patients who carried the BMP6 p.Leu96Pro mutation and did not share a unique haplotype, which may have resulted from multiple independent mutational events (Daher *et al.*, 2016). According to this, we did haplotype analysis, on the two chicken populations and found the haplotypes of BMP6 gene has obvious significance with partial skeletal traits and carcass traits, this consequence reminded us that BMP6 gene may have an effect of pleiotropism on chicken carcass traits and skeletal traits and in fact the genetic mechanisms among each characters are the function of pleiotropism. In this study, block 1 (CCGT, CCCT, GTCC, GTCT, GCCT) had no direct significance on neither carcass traits nor skeletal traits in Avian populations but was significant correlated on AFW, LW ($p<0.05$) in Yellow bantam populations. Block 2 (AAA, AGA, GGC, GGA) was significantly associated among EW, SEW BMW, LMW, TL ($p<0.01$) and also significantly affected LW, FW, TW, TD, FL ($p<0.05$) in Avian population. This result illustrated that AAA, AGA, GGC, GGA genotypes could choose to be

advantageous genotypes which promote carcass and skeletal traits of chicken. In Yellow bantam population, block 2 only correlated with FP significantly (*p*<0.05).

It can be concluded that there is difference between the Avian and Bantam yellow in various aspects. However, based on our experiment, the polymorphism in BMP6 gene in chicken causes little difference. Analyzing the reasons of all results, first, we sequenced partially of BMP6 sequence and BMP6 gene may have controlled growth and development of chicken in other ways. As a gene serves as bone morphogenetic, we may study BMP6 gene from a new angle of view to find its distinguished function in chicken.

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