Genetics and Molecular Research

Identification and characterization of *SREBF2* expression and its association with chicken carcass traits

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ABSTRACT. The sterol regulatory element-binding transcription factor 2 gene (*SREBF2*) plays an important role in regulating lipid homeostasis. To reveal the genetic factors that underlie carcass fat deposition in chickens, we cloned the coding DNA sequence of chicken *SREBF2*, investigated *SREBF2* mRNA expression levels in various tissues, detected single nucleotide polymorphisms (SNPs) in the exon regions of the gene, and conducted association analyses between single markers/haplotypes and carcass traits. The entire 2859-bp cDNA sequence of chicken *SREBF2* that encoded 952 amino acids was obtained and characterized. *SREBF2* mRNA was highly

expressed in the uropygial gland, followed by the liver, breast muscle, and leg muscle. Ten SNPs were detected, and four (g.49363077T>A, g.49357503C>T, g.49355533G>A, and g.49354641G>A) were novel. When analyzing the associations between the single mutations and carcass traits, significant differences were found in three SNPs and g.49357915G>A was highly significantly associated with most carcass traits, except for abdominal fat weight and sebum thickness. In addition, haplotype combinations that were constructed using the *SREBF2* SNPs were associated with breast muscle weight. Chickens with the combined genotype H21H21 had the highest live weight, carcass weight, eviscerated weight, and semi-eviscerated weight values. To the best of our knowledge, this is the first study conducted on chicken *SREBF2* polymorphisms, which are predictive of the genetics that underlie the economic performance of chickens.

Key words: Chicken; *SREBF2* gene; Cloning; Expression; Polymorphism

INTRODUCTION

Many factors influence chicken meat quality, including muscle development and tenderness and subcutaneous, abdominal, and intramuscular fat deposition. A suitable fat content can improve the quality of the chicken, but excessive fat deposition has many negative effects. With the continued improvement of living standards in China, the incidences of obesity, diabetes, and cardiovascular system diseases have also increased (Kaidar-Person et al., 2011; Reaven, 2011); therefore, dietary fat content is receiving an increasing amount of attention. Reducing body fat deposition by the regulation of poultry fat metabolism has become an important subject for many researchers.

Sterol regulatory element-binding proteins (SREBPs) belong to the nuclear transcription factors family and are important regulatory factors in animal body fat synthesis. Therefore, SREBP transcription factors are pivotal activators of key enzymes involved in cholesterol synthesis, low-density lipoprotein endocytosis, fatty acid synthesis, and glucose metabolism (Edwards et al., 2000; Zhao and Yang, 2012). There are three SREBP isoforms in mammals and birds: SREBP-1a, SREBP-1c, and SREBP2. SREBP-1a and SREBP-1c are encoded by the *SREBF1* gene, whereas SREBP2 is encoded by the *SREBF2* gene (Le Hellard et al., 2010). The transcriptional activity, tissue distributions, and modes of regulation of the SREBP-1a, SREBP-1c, and SREBF2 isoforms differ (Shimano et al., 1997; Bommer and MacDougald, 2011). Collectively, SREBPs can activate the transcription of virtually all of the genes involved in the synthesis of cholesterol, fatty acids, and phospholipids (Bommer and MacDougald, 2011).

Gene expression studies have revealed that SREBP-1a and -1c preferentially activate the transcription of genes involved in fatty acid synthesis, whereas SREBF2 is involved in cholesterol biosynthesis. At present, SREBF2 research is focused on mice and humans. For example, Yang et al. (2015) found that polymorphisms of *SREBF2* (rs1052717 and rs2267443) contribute to the underlying pathophysiology of metabolic syndrome in patients treated with clozapine. In studying the relationship between *SREBF2* and obesity and serum lipid levels in children and adolescents, Liu et al. (2014) found that carriers of GC/CC genotypes of the

SREBF2 rs2228314 polymorphism have a higher risk of abnormal high-density lipoprotein cholesterol levels than do individuals carrying the GG genotype. In the chicken, Zhang et al. (2014) found that the *SREBP2* expression level in the liver is highest at the 21days of embryonic stage. Therefore, based on previous research, we speculated that chicken *SREBF2* might play an important role in carcass fat deposition.

To the best of our knowledge, this is the first investigation of chicken *SREBF2* expression and polymorphisms. In this study, we isolated the full coding DNA sequence (CDS) of Erlang Mountain chicken *SREBF2* for the first time, analyzed its nucleotide sequence, investigated its expression levels in different tissues, and detected its sequence variants in 10 chicken populations; subsequently, we investigated the associations between the sequence variants and carcass traits. This study provides useful information on chicken genetics and breeding.

MATERIAL AND METHODS

Sample collection

The study was conducted in strict accordance with the requirements of the Animal Ethics Committee of Sichuan Agricultural University. The chickens that were involved in this study were humanely sacrificed to reduce suffering. Twenty Erlang Mountain chickens (10 hens and 10 cocks, 13 weeks of age) were provided by the poultry breeding farm of Sichuan Agricultural University and used to clone chicken *SREBF2* and for mRNA expression analysis. The chickens were randomly selected and slaughtered at the same time, and six fresh tissue types (liver, breast muscle, thigh muscle, abdominal adipose tissue, sebum cutaneum, and uropygial gland) were collected, immediately placed in liquid nitrogen, and stored at -80°C for RNA extraction.

To screen for single nucleotide polymorphisms (SNPs) and perform an association study, 120 Erlang Mountain chickens (including the SD02, SD03, SD01 x SD02, and SD01 x SD03 lines) and 180 high-quality Sichuan Daheng broilers (including the S01, S02, S03, S05, S06, and D99 lines) were randomly selected; all were 13 weeks old. During their growth period, all of the chickens had access to food and water *ad libitum*, were housed under the same temperature and light conditions, and their nutrition levels were completely consistent. After slaughter on the same day, live weight (LW), carcass weight (CW), eviscerated weight (EW), semi-eviscerated weight (SEW), breast muscle weight (BMW), leg muscle weight (LMW), abdominal fat weight (AW), and sebum thickness (ST) were measured. All of these performance traits were determined as described in "The Poultry Production Performance Terms and Measurement Statistics Method" (NY/T823-2004). Venous blood samples were taken from under the wings and prepared for DNA extraction.

RNA isolation and cDNA synthesis

Total RNA was extracted from the fresh tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol, and was then dissolved in RNase-free water. The integrity of the RNA was evaluated by electrophoresis on 1.0% agarose gels, and the concentration and purity of the RNA were measured using a NanoVue Plus™ spectrophotometer (Thermo Scientific, USA).

cDNA synthesis was performed in a volume of 10 μL with 1 μg total RNA using

a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China), according to the manufacturer instructions. The reaction conditions for cDNA synthesis were 37°C for 15 min followed by 85°C for 5 s and storage at 4°C.

Molecular cloning of Erlang Mountain chicken *SREBF2* cDNA

The predicted gene sequence of chicken *SREBF2* (XM_416222.2) was downloaded from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Sequences of all of the primers that were used in this study are listed in Table 1. The polymerase chain reaction (PCR) (total volume of 50 μL) contained 4.0 μL first-strand cDNA, 25 μL buffer, 8.0 μL dNTP, 0.5 μL LA *Taq* DNA polymerase (TaKaRa), 2 μL each primer (10 pmol), and 8.5 μL RNase-free H_2O. The optimum conditions for amplification were 10 min at 94°C, 35 cycles of denaturation at 94°C for 50 s, and annealing at 58°-63°C. The PCR products were subjected to electrophoresis on 1% agarose gels and purified using an E.Z.N.A.® Gel Extraction Kit (Omega, USA). The principal product was cloned into a pMD18-T vector (TaKaRa), and three randomly selected positive clones were sequenced by HuaDa Biotechnology Co. Ltd. (Beijing, China).

Table 1. Summary of the primers used in the study.

Usage	Name	Sequence (5'-3')	Annealing temperature (°C)	Fragment (bp)
Cloning	1	F: ATGTCCCGTGGAACCAACC	58.0	1453
		R:GATGGAGCTTGTGGTAGGC		
	2	F: AAGACGGACGGCAATC	61.5	2663
		R:TGAGGGCTGGTGAGGTGTTAG		
	3	F: ACCAGTGGAGGGCCAGAGA	63.0	1533
		R: ATGGAGGAAGTCCGGGCT		
RT-PCR	*SREBF2*	F: ACTCAATGGGAAGTGGAGCAC R: cactatgctgaaacgtgacctc	58	161
	β-actin	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	57.2	180
	F1	F: GGTCCAGCCTCAGATCATCAA R: TCCCCACCGTTAGAAA	55	230
	F2	F: GGCTGAATGCTGGTGACACTT R: TTACCTTGGCGTCTGT	53	267
SNP	F3	F: AACCCTGGAAGCACGTTGTAC R: CAATGATAAAGAACCGAAAG	55	244
	F4	F: GAACTGTTGAAGGGCATTGAC R: TGTGGCCCTTAAGTAACTCTA	55	230
	F5	F: TTTTCATCTCTCCGCACCAA R: ACTCGCAGCACCCCAACTCTT	60	248
	F6	F: TGCCCACGCTGATCCT R: TCCTCCTACGAGACGCATGTG	62	201
	F7	F: TAACCTTTCGCCACGATGTTT R: CGCTGGTCTTGGCCTCGTC	63	248
	F8	F: CCTCCAGCTCCGCTTAC R: TAGCAGAGGACGACACCGTGA	60	187
	F9	F: TCCTTTCCCCAGAGCTATTTC R: GGCTCCCAGGGCAAAGTACA	61	236
	F10	F: CACAAGTTCATCAGGCGTTCT R: AGACTAACCCGCACATT	56	196
	F11	F: CTTCTTGTTTCAGCGAGTTCT R: CATTTCCTGCAGCTAGTGG	56	165
	F12	F: ACCCGTGACTCCGTTCTTG R:TTTAGGAGCAGCACCACGCAC	60	176
	F13	F: CTGTGAAGGCACGGCTCT R: ATGGTGTGTAGCCCTTACGTT	60	216
	F14	F: CCTGGTGCTGAGCCGTGTCTG R: CCAAAGCTCACCTTGCGGTAC	64	235
	F15	F: CCTCCAGGTCTTCCTTCACGA R: CTACACAGCACCCAATAGCC	61	167
	F16	F: AGGGCAGAGGGAGCGAG R: AGGGCTGGTGAGGTGTTAGGA	62	217
	F17	F: GCCAGCGTGCCGTCCTG R: TCCCCAATTCCTTTTGCAACA	62	182

RT-PCR = reverse transcription-polymerase chain reaction; SNP = single nucleotide polymorphism.

Sequence analysis

The assembled cDNA sequence was evaluated by DNAMAN 6.0. Homologs were identified in GenBank using a tBLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search. The open reading frame (ORF) of chicken *SREBF2* was detected using the NCBI ORF-finder tool (http://www.ncbi.nlm.nih.gov/projects/gorf/). The phosphorylation sites of the SREBF2 protein were predicted by the NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos), and the presence and locations of signal peptides were predicted using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP). The transmembrane domain of the deduced amino acid (AA) sequence was predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0), and the secondary structures of the deduced AA sequence were predicted by HNN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html).

Expression analysis of chicken *SREBF2*

Total mRNA from the six chicken tissues was extracted to investigate the mRNA expression profiles of chicken S*REBF2* using real-time PCR. Each real-time PCR was conducted in triplicate. *β-actin* (housekeeping gene) was used as an internal control for each sample. The primers were designed according to the predicted mRNA sequence of chicken *SREBF2* (XM_416222.2) and the *β-actin* sequence (Table 1).

All of the reactions were performed in a CFX96 Real-Time PCR detection System (Bio-Rad, USA). Each reaction (total volume of 15 μL) contained 7.5 μL 2X SYBR® Premix Ex *Taq*™, 0.5 μL each primer, 1 μL normalized template cDNA from each tissue, and 9.5 μL sterile water. The reaction was performed as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 58°C for 25 s, and a final temperature increment of 0.5°C/s from 58 to 95°C. Chicken *SREBF2* mRNA expression was calculated relative to the amount of *β-actin* present.

The real-time PCR data were analyzed using the comparative Ct method (Schmittgen and Livak, 2008); Ct values are the means of the samples, which were tested in triplicate. Gene expression was calculated as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct$ = Ct target - Ct internal control), which indicates an n-fold difference relative to the expression of the internal control gene. The differential expression of *SREBF2* among the six tissues was analyzed by analysis of variance in SAS version 6.12 (Statistical Analysis Systems Institute Inc., Cary, NC, USA). Multiple comparison analysis was conducted using the Duncan test. Comparisons were considered significant at $P < 0.05$.

SNP scanning and genotyping

Seventeen pairs of primers (Table 1) were designed based on the *SREBF2* sequence (ENSGALG00000011916), and were synthesized by Shanghai Yingjun Biotechnology Co. Ltd. (Shanghai, China). The 10-μL reaction mix contained 5 μL 2X *Taq* PCR Master Mix (including Mg^{2+}, dNTPs, and *Taq* DNA polymerase; Beijing TIAN WEI Biology Technique Corporation, Beijing, China), 0.4 μL each primer, 0.8 μL DNA template (50 ng/mL), and 3.4 μL ddH_2O. The cycling protocol was as follows: 94°C for 4 min, 35 cycles at 94°C for 30 s, 58°C (or another appropriate annealing temperature, as shown in Table 1) for 30 s and 72°C for 1 min, with a final extension at 72°C for 8 min. Genetic variants in the *SREBF2* genomic sequence were analyzed using the PCR-single-strand conformation polymorphism

(SSCP) method. Briefly, after denaturation at 99°C for 10 min, 3 μL PCR product was rapidly cooled on wet ice and then loaded onto 16 x 18 cm, 12% acrylamide:bisacrylamide (39:1) gel. Electrophoresis was performed at 200-300 V for 13-15 h in 1X TBE buffer, and the gel was silver-stained. Three DNA samples that exhibited different patterns on the SSCP gel were further amplified and purified, and were then sequenced by the Shanghai Yingjun Biology Technique Corporation.

Statistical analysis

Genic and allelic frequencies were determined for each population by direct counting. The Hardy-Weinberg equilibrium (HWE), heterozygosity, homozygosity, and effective allele number were statistically analyzed according to the previous approaches of Nei and Roychoudhury (1974) and Nei and Li (1979). The polymorphism information content (PIC) was calculated according to Botstein et al. (1980)'s methods.

The linkage disequilibrium (LD) structure, as measured by *D*' and r^2, was constructed using the Haploview software version 3.32 (Barrett et al., 2005), and haplotypes were constructed using the PHASE program version 2.0 (Stephens et al., 2001). Association analyses between single SNP-marker genotypes and the carcass traits were performed using the general linear model procedure in SAS 6.12. The model used was as follows:

$$Y = \mu + B_i + S_j + G_k + B_i \times S_j \times G_k + e_{ijk} \quad \text{(Equation 1)}$$

where Y is the trait being measured; μ is the population mean; B_i is the fixed effect of breed; S_j is the fixed effect of sex; G_k is the fixed effect of genotype; B_i x S_j x G_k is the interaction among breed, sex, and genotype; and e is the random error. Values are reported as least square means ± SEM. Statistical significance was evaluated using the Duncan test, and differences were considered significant at $P < 0.05$.

RESULTS

Characterization of the chicken *SREBF2* sequence

The BLAST result from the NCBI's nucleotide sequence database revealed that Erlang Mountain chicken *SREBF2* was significantly similar to mammal *SREBF2* sequences. The Erlang Mountain chicken *SREBF2* CDS extended from position 1 to 2859 within the cDNA sequence, and encoded a 952-AA protein (Figure 1).

The deduced AA sequence's molecular weight was 103.76 kDa and its isoelectric point was 8.71. The chicken *SREBF2* sequence had 88 negatively charged residues (Asp + Glu) and 104 positively charged residues (Arg + Lys), which indicated that the protein should have an overall positive charge. Hydropathy correlation analysis revealed that the protein was highly hydrophilic. Fifty-eight phosphorylation sites were predicted by the NetPhos 2.0 server (Table 2); no signal peptides were identified by SignalP 4.0. The TMHMM results indicated that the protein had one transmembrane domain that was located between 17 and 33 AA. The secondary structure of the protein was predicted to be 46.95% α-helix, 43.70% random coil, and 9.35% extended strand (Figure 2).

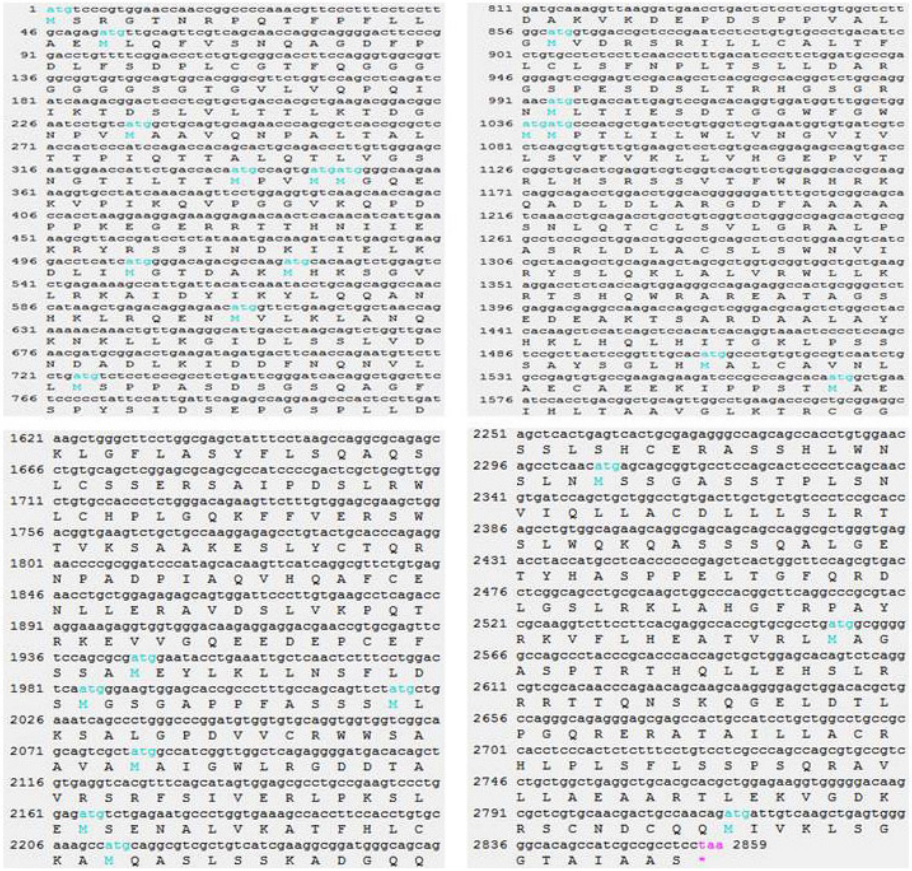

Figure 1. Nucleotide and deduced amino acid sequences of chicken *SREBF2*.

Table 2. Predicted chicken SREBF2 phosphorylation sites.

Amino acid acids Phosphorylation of amino acid	Locus position	Point score	Locus position	Point score	Locus position	Point score
Serine	155	0.995	322	0.959	661	0.509
	156	0.982	328	0.884	711	0.990
	222	0.987	379	0.898	719	0.993
	243	0.665	382	0.986	723	0.516
	247	0.948	422	0.972	743	0.987
	249	0.623	453	0.767	744	0.828
	256	0.991	465	0.979	754	0.981
	259	0.648	472	0.997	761	0.905
	262	0.791	494	0.631	774	0.823
	266	0.927	496	0.810	805	0.696
	280	0.980	521	0.597	857	0.914
	310	0.980	558	0.966	877	0.588
	317	0.996	567	0.895	905	0.651
	320	0.983	594	0.670	911	0.676
Threonine	5	0.888	462	0.702	704	0.799
	92	0.708	471	0.713	820	0.692
	111	0.728	586	0.919	873	0.605
	145	0.780	630	0.985	874	0.983
	923	0.584				
Tyrosine	596	0.693				

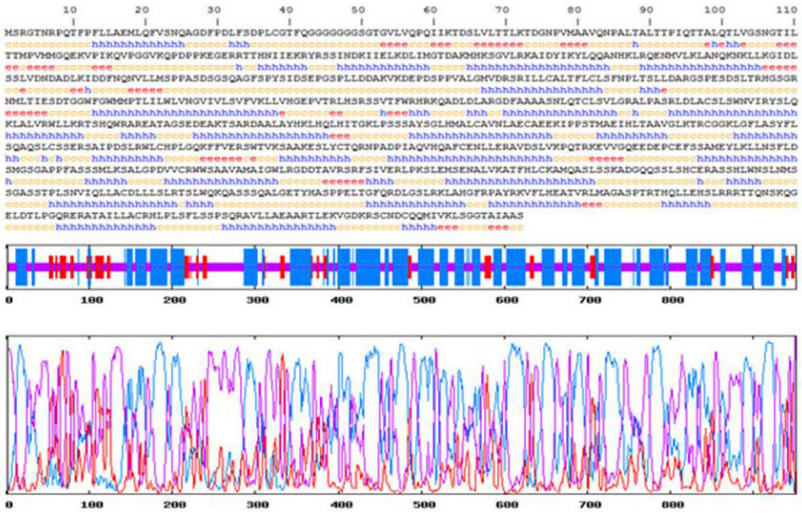

Figure 2. Schematic representation of the secondary structure of the chicken SREBF2 protein.

The deduced Erlang Mountain chicken *SREBF2* AA and gene sequences were compared with six *SREBF2* sequences from mammals and *Gallus gallus* using DNAMAN 6.0 (Table 3). The Erlang Mountain chicken *SREBF2* CDS was 99.8, 77.0, 76.2, 75.7, 78.2, 77.0, and 60.7% identical to that of *G. gallus*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Bos taurus*, *Canis lupus familiaris*, and *Danio rerio*, respectively. The deduced AA sequence of the Erlang Mountain chicken SREBF2 protein was 100, 82.4, 81.9, 81.7, 83.1, 82.3, and 53.8% identical to that of *G. gallus*, *H. sapiens*, *M. musculus*, *R. norvegicus*, *B. taurus*, *C. lupus familiaris*, and *D. rerio*, respectively.

Table 3. Similarity (%) of *SREBF2* mRNA and amino acid sequences.

Species	*Bos taurus*	*Canis lupus familiaris*	*Danio rerio*	Erlang Mountain chicken	*Gallus gallus*	*Homo sapiens*	*Mus musculus*	*Rattus norvegicus*
Bos taurus	-	93.7	52.6	83.1	83.1	91.7	91.3	91.2
Canis lupus familiaris	85.5	-	53.4	82.3	82.3	92.8	92.6	92.4
Danio rerio	56.1	55.7	-	53.8	53.8	52.9	52.7	52.9
Erlang Mountain chicken	78.2	77.0	60.7	-	100.0	82.4	81.9	81.7
Gallus gallus	75.5	76.4	59.8	99.8	-	82.4	81.9	81.7
Homo sapiens	84.6	86.5	56.1	77.0	76.3	-	92.7	92.9
Mus musculus	81.2	83.3	55.4	76.2	75.4	84.7	-	96.5
Rattus norvegicus	76.1	83.3	55.2	75.7	75.1	84.8	92.9	-

Similarity of nucleotide sequences are shown below the diagonal and similarity of amino acid sequences are shown above the diagonal.

Tissue expression patterns of chicken *SREBF2*

We evaluated the relative RNA expression levels of chicken *SREBF2* in different tissues using the quantitative PCR method (Figure 3). Statistical analysis demonstrated that there were highly significant differences in *SREBF2* transcript levels among the six tissues tested ($P < 0.01$). In 91-day-old chickens, *SREBF2* mRNA was most abundant in the uropygial gland, followed by the liver, breast muscle, and leg muscle, and was at extremely low levels in the sebum cutaneum and abdominal fat.

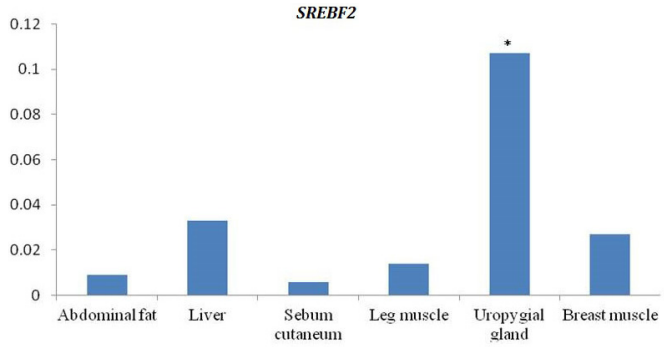

Figure 3. *SREBF2* expression in six tissues of the Erlang Mountain chicken as detected by quantitative polymerase chain reaction. Expression levels were normalized against *β-actin* and measured as $2^{(-\Delta\Delta Ct)}$ values. The results were averaged from three independent replicates that were measured at the 91-day-old stage. *Significant difference compared with other tissues.

Polymorphisms and genetic diversity

We investigated chicken *SREBF2* sequence variants by comparing our sequencing results with those of the chicken *SREBF2* sequence published in Ensemble (reverse-strand, Ensemble No. ENSGALG00000011916). Ten SNPs were identified; eight were in exons and two in introns. By comparing these results with those on the dbSNP database (http://www.ncbi.nlm.nih.gov/snp/), SNP1 (intron 6, g.49363077T>A), SNP5 (intron 12, g.49357503C>T), SNP7 (exon 14, g.49355533G>A), and SNP8 (exon 15, g.49354641G>A) were identified as novel SNPs, while the remaining SNPs were deposited as rs318011316 (SNP2, exon 7), rs13864629 (SNP3, exon 8), rs317877794 (SNP4, exon 11), rs10730451 (SNP6, exon 13), rs313438447 (SNP9, exon 16), and rs13864614 (SNP10, exon 19). Detailed information about the SNPs and AA changes are shown in Table 4. The genotyping of these SNPs was successfully performed using DNA sequencing and the PCR-SSCP method (Figure 4).

Table 4. Genetic variation in chicken *SREBF2*.

SNP	Position on chromosome	Location	Allele	AA exchange	Study result
SNP1	49363077	Intron 6	T>A	-	This study
SNP2	49362808	Exon7	C>T	-	rs318011316
SNP3	49361809	Exon8	T>C	-	rs13864629
SNP4	49357915	Exon11	G>A	-	rs317877794
SNP5	49357503	Intron12	C>T	-	This study
SNP6	49356963	Exon13	G>A	-	rs10730451
SNP7	49355533	Exon14	G>A	-	This study
SNP8	49354641	Exon15	G>A	-	This study
SNP9	49354218	Exon16	G>A	-	rs313438447
SNP10	49351627	Exon19	C>G	-	rs13864614

SNP = single nucleotide polymorphism; AA = amino acid.

Population genetic diversity parameters were estimated for the 10 populations (data not shown), and the statistical analysis revealed that A, C, A, A, C, G, G, G, and C alleles were predominant in the seven loci examined that contained SNP1, SNP2, SNP3, SNP4, SNP6, SNP7, SNP8, SNP9, and SNP10, respectively, in all 10 populations. Interestingly, unlike in the other populations, allele T was predominant at the SNP5 locus in population SD99. Four of the

SNPs (SNP3, SNP4, SNP5, and SNP7) in the 10 experimental lines were in HWE (P > 0.05) with a medium amount of genetic diversity (0.25 < PIC < 0.50). Three of the SNPs (SNP1, SNP2, and SNP10) were also in HWE (P > 0.05) but exhibited low genetic diversity (PIC < 0.25). However, SNP6 and SNP9 were not in HWE in the S01 and SD02 populations or the S05 and S06 populations, respectively (P < 0.05 or 0.01, respectively); the other populations had no SNP6 or SNP9 polymorphisms. Unlike the other mutation loci, SNP8 was in HWE in all of the populations except for S01 and S02 (P > 0.05), and the mean PIC was less than 0.1. Therefore, these three markers (SNP6, SNP8, and SNP9) were excluded from later analyses.

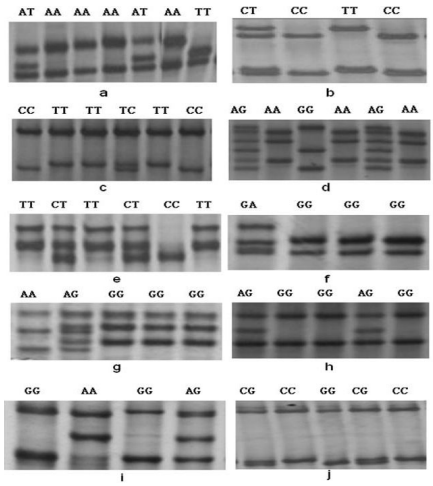

Figure 4. Polymerase chain reaction-single-strand conformation polymorphism patterns of *SREBF2* in the Erlang Mountain chicken. **a.** SNP1; **b.** SNP2; **c.** SNP3; **d.** SNP4; **e.** SNP5; **f.** SNP6; **g.** SNP7; **h.** SNP8; **i.** SNP9; **j.** SNP10.

Linkage and haplotype reconstruction of chicken *SREBF2*

D' and r^2 are the main parameters in LD analysis. Several researchers have found that r^2 is not as sensitive as *D*' to allelic frequency (Zhao et al., 2007; Marty et al., 2010). In cases where $r^2 > 0.33$, a sufficiently strong LD is available for mapping (Ardlie et al., 2002). The LDs of the seven SNPs in the 10 chicken populations were estimated, and the *D*' values ranged from 0.000 to 1.000 and the r^2 values from 0.000 to 0.320 (Table 5). Therefore, the results confirmed that the seven SNPs had little linkage disequilibrium.

Table 5. Estimated values of linkage disequilibrium between seven mutation sites in chicken *SREBF2*.

Locus	SNP1	SNP2	SNP3	SNP4	SNP5	SNP7	SNP10
SNP1		*D*' = 0.780	*D*' = 1.000	*D*' = 0.520	*D*' = 0.900	*D*' = 0.000	*D*' = 0.680
SNP2	r^2 = 0.320		*D*' = 0.121	*D*' = 0.020	*D*' = 0.820	*D*' = 0.000	*D*' = 0.254
SNP3	r^2 = 0.051	r^2 = 0.000		*D*' = 0.321	*D*' = 0.235	*D*' = 0.715	*D*' = 0.310
SNP4	r^2 = 0.040	r^2 = 0.000	r^2 = 0.031		*D*' = 0.740	*D*' = 0.721	*D*' = 0.334
SNP5	r^2 = 0.040	r^2 = 0.071	r^2 = 0.052	r^2 = 0.193		*D*' = 0.630	*D*' = 0.540
SNP7	r^2 = 0.012	r^2 = 0.030	r^2 = 0.164	r^2 = 0.050	r^2 = 0.126		*D*' = 0.710
SNP10	r^2 = 0.140	r^2 = 0.040	r^2 = 0.010	r^2 = 0.050	r^2 = 0.050	r^2 = 0.023	

Using the PHASE program, we found 30 haplotypes in the chicken populations tested (Table 6), the following four of which accounted for 49.79% of the estimates: H4 (ACCATAG),

H10 (ACTACGC), H13 (ACTATGC), and H16 (ACTGCGC). H13 (ACTATGC) had the highest frequency in all of the populations (14.52%), and the following six had frequencies of lower than 0.5%: H20 (ACTGTAC), H22 (ATTACGC), H23 (ATTATGC), H24 (TCTACGG), H28 (TTTATGG), and H29 (TTTGCGC).

Table 6. Haplotypes inferred based on seven single nucleotide polymorphisms.

Haplotype	Site							Frequency (%)
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP7	SNP10	
H1	A	C	C	A	C	G	C	2.86
H2	A	C	C	A	C	A	C	1.99
H3	A	C	C	A	T	G	C	7.57
H4	A	C	C	A	T	A	C	12.63
H5	A	C	C	A	T	A	G	0.67
H6	A	C	C	G	C	G	C	4.74
H7	A	C	C	G	C	G	G	3.07
H8	A	C	C	G	T	G	C	0.79
H9	A	C	C	G	T	G	G	0.98
H10	A	C	T	A	C	G	C	11.05
H11	A	C	T	A	C	G	G	4.08
H12	A	C	T	A	C	A	G	0.65
H13	A	C	T	A	T	G	C	14.52
H14	A	C	T	A	T	G	G	1.61
H15	A	C	T	A	T	A	C	0.81
H16	A	C	T	G	C	G	C	11.59
H17	A	C	T	G	C	G	G	2.54
H18	A	C	T	G	C	A	C	1.22
H19	A	C	T	G	T	G	C	1.50
H20	A	C	T	G	T	A	C	0.42
H21	A	T	C	A	C	G	C	5.58
H22	A	T	T	A	C	G	C	0.34
H23	A	T	T	A	T	G	C	0.41
H24	T	C	T	A	C	G	G	0.15
H25	T	C	T	G	C	G	C	0.92
H26	T	T	T	A	C	G	C	0.66
H27	T	T	T	A	C	G	G	0.83
H28	T	T	T	A	T	G	G	0.22
H29	T	T	T	G	C	G	C	0.34
H30	T	T	T	G	C	G	G	3.59

Associations between SNP markers and carcass traits

To investigate possible associations between the different genotypes and carcass traits, the effects of single markers on the carcass traits were analyzed (Table 7). Because breed was not significant, data from the 10 populations were pooled and analyzed together. For SNP1, the statistical analysis revealed that individuals with the genotypes AA or AT had significantly greater EW values than those with the genotype TT ($P < 0.01$), demonstrating that the A allele might be associated with increases in EW. However, the other traits evaluated had no significant associations with the genotypes ($P > 0.05$). For SNP2, chickens with CC or CT genotypes had greater EW values than those with the TT genotype ($P < 0.05$), but the other traits evaluated had no significant associations with any genotypes ($P > 0.05$). For SNP4, individuals with the AA genotype had higher values for the following carcass traits than individuals with AG or GG genotypes ($P < 0.01$): LW, CW, EW, SEW, BMW, and LMW. However, the other traits evaluated had no significant associations with any genotypes ($P > 0.05$). For the remaining markers (SNP3, SNP5, SNP7, and SNP10), none of the carcass traits had significant associations with the genotypes in any of the populations (data not shown) ($P > 0.05$).

Table 7. Associations between *SREBF2* single loci and chicken carcass traits.

SNP	Genotype	LW (g)	CW (g)	SEW (g)	EW (g)	BMW (g)	LMW (g)	AW (g)	ST (cm)
SNP1	AA	2481.63 ± 572.52	2202.21 ± 501.88	2059.52 ± 479.26	1683.93 ± 377.43^{A}	139.80 ± 34.35	192.58 ± 52.48	71.89 ± 44.08	0.49 ± 0.17
	AT	2461.43 ± 504.92	2195.00 ± 448.37	2051.11 ± 428.08	1675.30 ± 342.15^{A}	139.21 ± 32.79	191.79 ± 50.10	65.80 ± 37.75	0.48 ± 0.19
	TT	3110.00 ± 636.39	2807.50 ± 611.65	2634.80 ± 544.19	1925.00 ± 460.10^{B}	179.02 ± 23.79	248.10 ± 82.72	80.72 ± 30.72	-
SNP2	CC	2438.01 ± 557.09	2167.04 ± 494.87	2025.63 ± 471.84	1661.62 ± 373.42^{a}	136.96 ± 33.31	189.91 ± 51.74	70.43 ± 44.56	0.50 ± 0.17
	CT	2586.44 ± 550.44	2289.47 ± 465.29	2141.88 ± 446.04	1699.60 ± 385.08^{a}	147.57 ± 34.34	197.34 ± 49.84	74.53 ± 39.35	0.47 ± 0.17
	TT	3047.50 ± 560.48	2708.13 ± 497.93	2551.65 ± 468.30	2064.11 ± 388.53^{b}	172.20 ± 37.23	247.70 ± 64.53	66.24 ± 30.96	-
SNP4	AA	2538.69 ± 568.57^{A}	2264.44 ± 511.99^{A}	2122.94 ± 486.78^{A}	1734.51 ± 376.93^{A}	143.91 ± 35.45^{A}	201.12 ± 53.07^{A}	73.15 ± 45.68	0.49 ± 0.18
	AG	2428.47 ± 590.46^{B}	2147.12 ± 502.88^{B}	2003.80 ± 482.27^{B}	1623.12 ± 397.43^{B}	135.97 ± 33.95^{B}	185.95 ± 53.75^{B}	68.02 ± 39.36	0.50 ± 0.15
	GG	2453.51 ± 423.46^{AB}	2173.38 ± 376.11^{A}	2025.16 ± 354.09^{AB}	1657.52 ± 295.00^{AB}	138.41 ± 27.87^{AB}	183.94 ± 37.93^{B}	74.57 ± 46.72	0.51 ± 0.19

Least square mean values within a row of the same SNP locus with different lowercase superscript letters differed significantly at $P < 0.05$; least square mean values within a row of the same SNP locus with different uppercase superscript letters differed significantly at $P < 0.01$. SNP = single nucleotide polymorphism; LW = live weight; CW = carcass weight; SEW = semi-eviscerated weight; EW = eviscerated weight; BMW = breast muscle weight; LMW = leg muscle weight; AW = abdominal fat weight; ST = sebum thickness.

Associations between combined genotypes and carcass traits

Haplotype analysis can provide more information than single-marker analysis on genetic diseases and trait associations because of a population's ancestry and demography (Akey et al., 2001). The advantage of using haplotype-based methods is greatest when marker alleles are not in strong LD with each other (Morris and Kaplan, 2002). In the present study, all 30 of the haplotypes evaluated were used for establishing combinations, and 117 haplotype combinations were identified. These combinations were selected for further analysis, except those with percentages lower than 1%. Data of the associations between haplotypes and carcass traits are shown in Table 8.

Table 8. Associations between combined *SREBF2* genotypes and chicken carcass traits.

Combined genotype	Traits							
	LW (g)	CW (g)	SEW (g)	EW (g)	BMW (g)**	LMW (g)	AW (g)	ST (cm)
H1H4	2553.33 ± 412.88	2270.00 ± 377.46	2116.62 ± 264.35	1717.27 ± 175.62	141.83 ± 17.72	202.91 ± 34.90	73.87 ± 16.52	0.569 ± 0.208
H3H4	2855.00 ± 332.58	2541.43 ± 362.78	2371.89 ± 234.95	1920.91 ± 206.96	170.72 ± 21.97	226.85 ± 38.96	94.52 ± 28.63	0.450 ± 0.035
H3H6	2446.25 ± 477.20	2191.25 ± 250.95	2019.08 ± 286.37	1661.09 ± 269.15	145.96 ± 22.74	193.14 ± 30.41	63.56 ± 18.01	0.412 ± 0.160
H3H10	2401.25 ± 543.02	2137.50 ± 218.58	2020.40 ± 209.67	1674.52 ± 242.34	137.77 ± 27.24	190.59 ± 32.36	78.08 ± 22.97	0.558 ± 0.183
H3H11	2555.00 ± 357.03	2291.67 ± 328.60	2170.90 ± 339.15	1779.43 ± 305.35	135.85 ± 26.94	208.58 ± 27.94	63.82 ± 23.45	0.416 ± 0.090
H3H13	1995.00 ± 164.22	1775.00 ± 154.11	1637.50 ± 161.37	1381.25 ± 129.70	100.70 ± 6.59	161.53 ± 18.10	38.85 ± 9.13	0.484 ± 0.155
H3H16	2333.33 ± 470.62	2080.67 ± 233.33	1959.96 ± 214.72	1635.98 ± 217.20	126.36 ± 20.32	186.34 ± 23.28	48.43 ± 19.23	0.476 ± 0.126
H4H10	2670.00 ± 411.60	2405.67 ± 200.61	2236.75 ± 260.93	1800.48 ± 272.16	155.31 ± 26.39	207.13 ± 36.58	84.20 ± 26.23	0.569 ± 0.173
H4H11	2804.00 ± 390.61	2517.00 ± 200.93	2369.18 ± 268.63	1987.21 ± 194.94	154.83 ± 21.51	248.54 ± 26.34	48.70 ± 14.71	0.464 ± 0.251
H4H13	2376.00 ± 336.46	2135.50 ± 296.64	2006.85 ± 278.50	1639.27 ± 171.45	130.07 ± 25.78	181.99 ± 22.44	79.83 ± 27.52	0.465 ± 0.197
H4H16	2477.17 ± 620.77	2210.00 ± 246.58	2071.56 ± 225.96	1690.16 ± 209.52	128.94 ± 26.38	191.09 ± 34.96	70.94 ± 27.15	0.437 ± 0.103
H4H17	2433.33 ± 364.60	2175.00 ± 336.34	1958.33 ± 267.93	1675.00 ± 254.44	141.47 ± 25.02	201.37 ± 28.63	54.00 ± 27.23	0.477 ± 0.116
H4H21	2739.29 ± 479.90	2457.86 ± 234.22	2337.72 ± 230.05	1906.72 ± 218.77	166.25 ± 28.45	214.45 ± 32.78	67.46 ± 23.36	0.401 ± 0.084
H6H16	2410.00 ± 236.78	2162.50 ± 208.67	2050.00 ± 188.19	1681.25 ± 139.01	135.20 ± 11.70	196.13 ± 14.13	72.25 ± 26.26	0.513 ± 0.226
H7H10	2870.00 ± 368.16	2546.25 ± 366.74	2385.80 ± 334.81	1970.15 ± 323.07	167.30 ± 21.59	208.85 ± 36.38	60.68 ± 21.63	0.444 ± 0.007
H7H13	2499.50 ± 635.52	2211.98 ± 296.42	2057.92 ± 259.51	1684.41 ± 224.66	137.50 ± 21.44	191.23 ± 37.17	78.93 ± 24.26	**0.655 ± 0.208**
H10H10	2651.67 ± 646.02	2337.50 ± 246.59	2178.67 ± 213.92	1776.18 ± 262.45	147.99 ± 27.26	199.67 ± 24.62	**109.50 ± 21.23**	0.579 ± 0.179
H10H13	2180.71 ± 408.97	1947.50 ± 185.22	1828.72 ± 164.26	1494.19 ± 270.59	121.86 ± 24.48	167.13 ± 22.76	81.80 ± 21.27	0.551 ± 0.188
H10H16	2659.29 ± 494.79	2244.29 ± 304.78	2084.63 ± 288.39	1712.68 ± 277.18	138.50 ± 22.23	202.62 ± 33.55	78.33 ± 20.19	0.538 ± 0.135
H11H13	2593.75 ± 432.06	2313.75 ± 362.15	2178.01 ± 316.39	1785.91 ± 252.48	161.28 ± 27.46	213.35 ± 42.95	55.22 ± 13.20	0.442 ± 0.058
H13H13	2552.14 ± 448.29	2247.14 ± 327.82	2097.66 ± 363.68	1714.60 ± 270.62	149.77 ± 28.04	186.74 ± 30.83	78.15 ± 25.58	0.443 ± 0.133
H13H16	2244.23 ± 474.45	1987.69 ± 238.15	1849.20 ± 215.29	1489.79 ± 201.08	126.48 ± 21.32	166.12 ± 26.52	71.61 ± 27.23	0.511 ± 0.108
H13H17	2535.71 ± 330.23	2242.14 ± 227.82	2104.00 ± 203.92	1719.92 ± 204.67	145.85 ± 29.74	195.89 ± 30.44	92.40 ± 32.65	0.607 ± 0.112
H13H21	2890.00 ± 342.69	2517.86 ± 340.04	2395.48 ± 317.10	1926.37 ± 267.14	**173.78 ± 27.80**	230.23 ± 31.44	101.28 ± 27.75	-
H13H30	2516.00 ± 423.12	2227.00 ± 377.47	2082.88 ± 336.65	1688.76 ± 282.34	152.81 ± 29.54	170.27 ± 30.59	105.62 ± 17.46	0.588 ± 0.111
H16H16	2416.67 ± 247.20	2179.17 ± 235.81	2029.17 ± 225.51	1695.83 ± 207.01	144.87 ± 22.96	178.97 ± 30.29	97.12 ± 33.46	0.652 ± 0.167
H21H21	**3115.00 ± 358.71**	**2770.00 ± 266.85**	**2603.73 ± 254.35**	**2090.78 ± 267.71**	172.67 ± 26.41	**257.38 ± 34.31**	90.52 ± 8.63	-

**Significant difference between least mean squares at $P < 0.01$, respectively. LW = live weight; CW = carcass weight; EW = eviscerated weight; SEW = semi-eviscerated weight; BMW = breast muscle weight; LMW = leg muscle weight; AW = abdominal fat weight; ST = sebum thickness. Underlined values present the lowest value and the bolded values present the highest value.

Chickens with the combined genotype H13H21 had the highest BMW and those with the combined genotype H3H13 had the lowest BMW, and all of the combined genotypes were

significantly associated with this trait (P = 0.006). The combined genotype H21H21 had the highest LW, CW, EW, and SEW values, H13H21 had the highest BMW value, H10H10 had the highest AW value, and H7H13 had the highest ST value. H3H13 had a negative effect on LW, CW, EW, SEW, BMW, LMW, and AW.

DISCUSSION

The *SREBF2* nucleotide sequence alignment results revealed that the Erlang Mountain chicken *SREBF2* CDS was similar to that of most mammals. We found one transmembrane domain in Erlang Mountain chicken *SREBF2*; however, Brown and Goldstein (1997) reported *SREBF2* as having two transmembrane domains.

The mRNA expression levels found in this study were consistent with those reported by previous studies. Assaf et al. (2003) reported chicken *SREBF2* as being highly expressed in the uropygial gland and liver, with comparatively lower expression levels in adipose tissue and skeletal muscle. In addition, Gondret et al. (2001) found that *SREBF2* expression levels in pig, rabbit, and chicken livers were twice as high as those in adipose tissue. Interestingly, we found that Erlang Mountain chicken *SREBF2* was highly expressed in breast muscle, suggesting that *SREBF2* might play an important role in meat quality. Whether the SREBF2 protein also regulates muscle fiber growth in chickens is unclear. Further studies of the function of the SREBF2 protein in the Erlang Mountain chicken are warranted to determine its role in the growth of muscle fiber.

Marker-assisted selection is a more accurate and convenient method of selection than traditional selection. Therefore, in the present study, the genomic DNA sequences of 10 chicken populations were successfully amplified using primer pairs for *SREBF2*. Based on previously reported sequences (Ensemble number: ENSGALG00000011916), 10 SNPs were identified in chicken *SREBF2* by sequencing, four of which were novel mutations. We combined the DNA sequencing results with those generated by the PCR-SSCP method, which accurately detected SNPs in chicken *SREBF2*.

At the SNP6 and SNP8 loci, mutation homozygotes (SNP6-TT and SNP8-AA) were not detected in any of the chicken populations studied. At the SNP1, SNP2, SNP9, and SNP10 loci, the mutation homozygotes TT, TT, AA, and GG were not found in the S01, S02, S03, S06, D99, SD02, or SD03 populations. This demonstrates that frequencies of T, A, T, T, A, and G alleles in the chicken populations decreased during artificial selection, migration, and genetic drift, possibly because these alleles may be negative mutations in certain chicken populations, or through natural selection, individuals with the genotypes that were eliminated caused a decline in the number of T, A, T, T, A, and G alleles in this study. However, the reason why mutations of the homozygotes mentioned above were absent in these chicken populations still needs further investigation.

Haplotype frequencies and LD coefficients were assessed for seven SNPs (SNP6, SNP8, and SNP9 were excluded) in all 10 chicken populations. The D' and r^2 values indicated that the seven SNPs in this study had little LD. Based on these results, 30 haplotypes were identified in the chicken populations. Haplotype H13 (ACTATGC) had the highest frequency in the population (14.52%). H20 (ACTGTAC), H22 (ATTACGC), H23 (ATTATGC), H24 (TCTACGG), H28 (TTTATGG), and H29 (TTTGCGC) had frequencies lower than 0.5%. The high-frequency haplotypes probably existed in the population for a long time. Novel variations are derived from common haplotypes, and rare variants represent mutations that are

more recent and are more likely to be related to common haplotypes than to other rare variants (Huang et al., 2013; He et al., 2014).

To evaluate the effects of the seven SNPs on carcass traits, an association analysis between single SNP genotypes, haplotype combinations, and the carcass traits was conducted, which revealed that SNP4 in chickens with the AA genotype resulted in more desirable LW, CW, EW, SEW, BMW, and LMW values. SNP2, SNP3, SNP4, SNP7, and SNP10 were synonymous mutations that did not cause AA variations, and SNP1 and SNP5 were in introns. However, it has recently been found that silent mutations can affect gene function and phenotype (Ren et al., 2014; Wang et al., 2014). Four silent mutations (g.69307744C>T, g.69355665T>C, g.69340192G>A, and g.69340070C>T) of chicken *TBC1D1* are significantly associated with carcass traits (Wang et al., 2014). One synonymous mutation (g.T1694A) in exon 4 of cattle *CFL2* is significantly associated with growth traits in Qinchuan cattle (Sun et al., 2015). Therefore, it would be interesting to determine the mechanism of the association between these silent mutations and carcass traits in chickens.

Combined genotypes (diplotypes) determine the usefulness of employing closely linked markers to identify genetically superior individuals, and are an essential component of genetic architecture (Stirling and Stear, 2010). To investigate the effects of combined genotypes on carcass traits, we analyzed the combined genotypes present in the chicken populations. The results revealed that the combined genotype H21H21 had the highest LW, CW, EW, and SEW values, H13H21 had the highest BMW value, H10H10 had the highest AW value, and H7H13 had the highest ST value. Therefore, our data demonstrate that associations between combined genotypes and carcass traits are more accurate than those between single SNP genotypes and carcass traits. These results are similar to those of Fallin et al. (2001) and He et al. (2014), who demonstrated that the inheritance of genotype combinations is more effective than that of a single SNP genotype. Therefore, H21H21 may be used as a molecular marker of combined genotypes in the future for the selection of desirable chicken carcass traits.

In summary, this is the first analysis of *SREBF2* polymorphisms in the chicken. Ten *SREBF2* SNPs were validated in Erlang Mountain and Sichuan Daheng chicken populations, three of which were significantly associated with LW, CW, EW, SEW, BMW, and LMW. Thirty haplotypes were identified, and the combined genotype H21H21 had the highest LW, CW, EW, and SEW values while H13H21 had the highest BMW value. Quantitative PCR data suggest that chicken *SREBF2* may play a role in muscle development. Therefore, our results suggest that *SREBF2* could be used as a DNA molecular marker of chicken carcass traits in marker-assisted selection.

Conflicts of interest

The authors declare no conflict of interest.

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