Investigation of porcine candidate genes for improving marbling score and meat quality traits in commercial swine breeds in Thailand

W. Kenchaiwong^{1,3}, S. Kananit^{2,3}, K. Tuangsithanon⁴, M. Duangjinda^{2,3}
and W. Boonkum^{2,3}

¹ Faculty of Veterinary Science, Mahasarakham University, Mahasarakham, Thailand

² Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand

³ Network Center for Animal Breeding and OMICS Research, Khon Kaen University, Khon Kaen, Thailand

⁴ Betagro Hybrid International Co., Ltd., Bangkok, Thailand

Corresponding author: W. Boonkum
E-mail: wboonkum@gmail.com

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ABSTRACT. The objective of this research was to identify candidate gene markers based on their function related to marbling score and meat quality traits in pigs. A total of 313 commercial pigs with five different Duroc (DR) breed fractions: 1 = 100%DR (n = 227), 2 = 50%DR x 50% Large white (LW)(n = 7), 3 = 50%DR x 50%Landrace (LR)(n = 41), 4 = 75%DR x 25%LW (n = 18), and 5 = 50%DR x 25%LW x 25%LR (n = 20) were measured for marbling score (score 3; MB3 and score 6; MB6), Color (L*), % cooking loss and shear force meat characters. The pigs were slaughtered at around 190 days of age, at an average weight of 122 kg. Five candidate genes (*LIPE*, *LEPR*, *ADRB3*, *CAPN1*, and *CAST*) were genotyped by using -RFLP. The genetic parameters were estimated with Bayesian analysis via Gibbs sampling and analyzed by estimated breeding value by BLUP. The breed group had an effect on traits, especially marbling traits, percent cooking loss, and shear force. In Duroc purebreds, the marbling scores were significantly higher than in Duroc crossbreds. All parameters were significantly higher in 50%DR

x 50% Large white (LW) compared with the others. Association analysis showed that the AG genotype of the *ADRB3* gene had a higher marbling score than the GG genotype (2.22 vs 1.99 for MB3, 2.92 vs 2.49 for MB6; $P < 0.05$). The lowest estimated breeding values (EBVs) for color (L^*)(3.88) and % cooking loss (2.64) were obtained in the CC genotype of the *CAPN1* gene. The GG genotype showed lower shear force and EBVs compared to the AG genotype of *CAST* gene (4.08 vs 4.85 for shear force ($P < 0.05$) and -0.03 vs 0.05 for EBV's, $P < 0.01$). The AG genotype of *ADRB3* could be used in marker assisted selection to improve marbling scores. Also, the GG genotype of *CAST* is the most appropriate for selection for pork tenderness.

Key words: Pigs; Gene association; Meat quality; Marbling score

INTRODUCTION

Meat quality is one of the key factors for consumers in purchasing decisions. The characteristics, i.e. color, juiciness, flavor, and tenderness, can affect consumer acceptance and are important in the competitive market of pig production (Fortin et al., 2005; Reardon et al., 2010). In addition, intramuscular fat and fatty acid composition has been shown to influence human health (Zhang et al., 2009; Xue et al., 2015). The candidate gene approach is used for difficult traits such as carcass and meat quality, sex and/or traits which show low heritability. The function of genes is frequently considered for candidate markers. The molecular markers can be applied with marker assisted selection (MAS) and/or for pre-selection in a breeding program. The polygene on the chromosomal regions known as quantitative trait loci (QTL) play a role in controlling meat quality traits (Cho et al., 2015), backfat thickness and intermuscular fat content (de Koning et al., 1999) in pigs. Several QTL markers for meat quality were detected in various populations. Linkage disequilibrium (LD) markers that can be transmitted within the population are frequently detected in the Duroc population (Gerbens et al., 1999; Cho et al., 2015). Currently, QTLs suitable for mapping, which can identify the major genes related to meat quality in term of fatness, are already known. Beta-3-adrenergic receptor gene (*ADRB3*) is responsible for encoding a major lipolytic and thermogenic mediator, which has an effect on adipose tissue. Nowacka-Woszuk et al. (2008) reported that the *ADRB3* gene is close to a quantitative trait locus (QTL) region for fatness traits on chromosome 15. The hormone-sensitive lipase gene (*LIPE*) on chromosome 6 is expressed in adipose tissue and plays a role in hydrolyzing triglycerides for free fatty acid mobilization (Mellink et al., 1993; Miyoshi et al., 2008; Zechner et al., 2009). Polymorphisms of the *ADRB3* and *LIPE* genes could be associated with the exhibition of intramuscular fat (IMF) in pigs, as reported by Xue et al. (2015). With regard to tenderness, several studies found that the calpain genes, i.e. calpain 1 and calpain 3, play important roles in tenderization processes (Huff-Lonerger and Lonergan, 2005; Gandolfi et al., 2011). Calpain 1 (*CAPN1*) encodes the large subunit (μ -calpain) of an enzyme which plays a role in proteolysis processes in pork (Huff-Lonerger and Lonergan, 2005; Kemp et al., 2010). High calpain activity leads to enhanced tenderness because it affects the cleavage of specific myofibrillar proteins (Gandolfi et al., 2011). Some studies

have reported that calpain 3 could not be detected in pig meat (Parr et al., 1999), whereas others have stated that the porcine calpastatin (*CAST*) is an antagonist of calpain, which means that high activity leads to decreased proteolysis and increased meat toughness (Kent et al., 2004; Kemp et al., 2010). Furthermore, the leptin receptor gene (*LEPR*) is associated with tenderness in pork, as reported by Wang et al. (2013). Providing beneficial information for marker-assisted selection in breeding programs will improve pig production in Thai swine crossbreeds. Therefore, the objective of this research was to identify candidate gene markers based on their gene function related to meat quality traits in pigs.

MATERIAL AND METHODS

Animals and sample collection

A total of 313 commercial pigs were split into 5 groups based on five Duroc (DR) breed fractions, including 1 = 100%DR (n = 227), 2 = 50%DR x 50%Large white (LW)(n = 7), 3 = 50%DR x 50% Landrace (LR)(n = 41), 4 = 75%DR x 25%LW (n = 18), and 5 = 50%DR x 25%LW x 25%LR (n = 20). The pigs were reared under evaporative cooling housing and under the same management conditions. A total of 313 pigs were randomly sampled for genomic DNA extraction and genotyping. The initial live body weights of sampled pigs ranged from 30-60 kg, and they were reared in common pens to observe the growth performance. Pigs were fed *ad libitum* with the same rations as the testing station, and the individual feed intake and body weight were recorded using feed intake recording equipment. The pigs were slaughtered at around 190 days of age (140-238 days). The meat quality traits of pigs, such as marbling score, color (L*), % cooking loss and shear force, were recorded when the slaughter weight of 88-160 kg was reached. Marbling scores were detected by standard industry score typing and assessed using a scale from 1 to 6. Our research specified the score typing as 1 to 3 for suitability in Thailand where the grouping scores 2-3 and 4-6 form the standard industry score typing. Only a general indication of lightness of meat color (L*) was determined with a colorimeter. Cooking loss was the differential of sample weights before and after incubation at 75°C for 10 min. Warner-Bratzler probes were used to measure shear force.

PCR-RFLP genotyping

The primer characteristics of each candidate gene are shown in Table 1. The restriction fragments of each candidate gene were amplified in a total reaction volume of 10 µL containing: 1 µL of genomic DNA, 1.5 µL of 10 X buffer with MgCl₂, 1 µL of dNTPs, 1 µL of each primer and 0.25 U of Taq Polymerase. The PCR protocol consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 60-66°C for 30-45 s based on annealing temperature of each gene (Table 1), and 72°C for 60 s, and a final elongation step at 72°C for 1.5 min. Genomic DNA fragments were digested by restriction endonuclease enzymes (incubated overnight at 37°C) and the digested fragments were separated by 2% agarose gel electrophoresis. All gene polymorphisms were identified by the Gel Documentary system.

Table 1. Primers and restriction enzymes in each candidate gene for marbling score and meat quality traits in purebred and crossbred Duroc pigs.

Candidate genes ⁵	5'-sequence-3' Forward / Reverse primer	AT. (°C) ⁴	Res. Enz. ⁶	PCR Sizes (bp)	Res. Enz. production sizes (bp)
<i>ADRB3</i> ¹	CGTTCAACCCGCTCATCTACTGC/ GTTCCCTACTCTGTGCCCCGTCTT	66	TaqI	315	A=315 G=143, 172
<i>LIPE</i> ²	CGCACAATGACACAGTCACTGGT/ AGGCAGCGGCCGTAGAAGCA	66	BsaHI	497	A=307, 190 G=240, 190, 67
<i>LEPR</i> ¹	TGCCTGCTGGAATCTCAAAG/ TTCCCTGCAATGTTGTCTGC	66	Tsp509I	184	A=184 G=113, 71
<i>CAST</i> ³	AATGAGCAGCCAAACATCAGA/ TTCCCATAGCCCACAAGAAG	60	HinfI	376	A=376 G=129, 247
<i>CAPNI</i> ³	CTGCTGCCCCACCAAGAC/ CTCGTAGCTGCCATTACC	60	HhaI	197	T=132, 65 C=91, 65, 41

¹Wang et al. (2013), ²Xue et al. (2015), ³Gandolfi et al. (2011), ⁴Annealing temperatures, ⁵*LIPE*=Lipids E hormone, *LEPR*=leptin receptor, *ADRB3*=adrenoceptor beta 3, *CAPNI*=Calpain, *CAST*=calpastatin gene, ⁶Restriction enzymes.

Statistical analysis

Genetic parameter estimates

Single trait via animal models was used for estimated variances and heritability, while genetic correlations were estimated by a bivariate threshold linear model between marbling score (threshold trait) and linear traits, as shown in equation 1. The genetic parameters were estimated with Bayesian analysis via Gibbs sampling by using the THRGIBBS1F90 program and G0+93

We used the Gibbs1f90 program developed by Tsuruta and Misztal (2006). The details in posterior distribution, with a Gibbs chain of 300,000 cycles were analyzed. The first 50,000 were used for burn-in samples. The autocorrelation was less than 0.25 and saved only one sample from every 50 iterations. The best linear unbiased prediction (BLUP) was used for estimated breeding value (EBV) analysis.

$$\begin{bmatrix} \delta \\ y \end{bmatrix} = \begin{bmatrix} X_1 & 0 \\ 0 & X_2 \end{bmatrix} \begin{bmatrix} \beta_1 \\ \beta_2 \end{bmatrix} + \begin{bmatrix} Z_1 & 0 \\ 0 & Z_2 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \end{bmatrix} \quad (\text{Eq. 1})$$

Assumption of α and ε is

$$\text{var} \begin{bmatrix} \alpha \\ \varepsilon \end{bmatrix} = \begin{bmatrix} G \otimes A & 0 \\ 0 & R \otimes I \end{bmatrix} \quad (\text{Eq. 2})$$

Where δ and y is the vector of observations of threshold traits and continuous traits, β is the vector of fixed effects, α is the vector of additive genetic effects, ε is vector of residuals, A is the additive genetic relationship matrix between animals, X_i and Z_i are the incidence matrices for fixed and random effects of traits, respectively, and i , G and R are the (co)variance matrices of vectors α and ε .

Association analysis

Genotype frequencies were estimated as per the formula of Falconer and Mackay (1996). A linear mixed model was used to analyze the association between each gene and traits i.e. marbling score (MB), Color (L*), % cooking loss, and shear force. Breed groups,

gender, genotype and covariate of slaughter weight were considered fixed effects, while sire was considered a random effect in the equation (2) and association with EBV was detected by equation (3).

$$y_{ijk} = \mu + Sex_i + Gene_j + \beta_0(Wt) + Sire_k + \varepsilon_{ijk} \quad (\text{Eq. 3})$$

$$EBV_{ij} = \mu + Gene_j + \varepsilon_{ij} \quad (\text{Eq. 4})$$

Where, y_{ijk} and EBV_{ij} are the phenotypic and estimated breeding value of traits, μ is the overall mean, Sex_i is the effect of sex i , $Gene_j$ is the effect of genotype of gene j , $\beta_0(Wt)$ is the regression of the slaughter weight (w_t) of test, $Sire_k$ is the random effect of sire k and ε_j is the residual error.

RESULTS AND DISCUSSION

The marbling scores are shown in Figure 1. The frequencies of each score in this population were 28, 30, 23, 11, 4 and 4% for scores 1 to 6, respectively.

Figure 1 Marbling score from 1 to 6 in Thai crossbred pigs

The effect of breed groups on marbling and meat quality traits

The breed group has an effect on traits, especially marbling traits, percent cooking loss, and shear force (Table 2). In Duroc purebreds, it appears that the marbling score were higher than in Duroc crossbreds ($P < 0.01$). Meanwhile, all parameters were significantly higher in 50%DR x 50% Large white (LW) compared with the others. This result is similar to what was found by Ellis et al. (1996), who reported that the Duroc purebred has good

meat quality and is highly marbled. Moreover, the Duroc crossbred (Shanzhu × Duroc) has good meat and fatness traits, as determined by the IMF content and fatty acid composition.

Table 2. The mean ± SE to test effect of breed groups on marbling scores and meat quality traits of marbling score and meat quality traits in purebred and crossbred Duroc pigs.

Traits ²	Breed groups ¹					P-value
	100%DR	50%DR x 50%LW	50%DR x 50%LR	75%DR x 25%LW	50%DR x 25%LW x 25%LR	
MB6	2.63±0.27 ^b	1.88±0.29 ^{ac}	2.11±0.46 ^{abc}	2.02±0.19 ^c	1.15±0.07 ^a	<.0001
MB3	2.01±0.04 ^b	1.61±0.15 ^c	1.85±0.24 ^{bc}	1.70±0.10 ^c	1.15±0.14 ^a	<.0001
Color (L*)	44.76±0.09	45.38±0.33	44.98±0.52	44.68±0.21	44.88±0.31	0.450
Cooking loss (%)	28.39±0.10 ^b	29.62±0.33 ^c	28.10±0.53 ^b	28.62±0.22 ^b	28.77±0.32 ^{abc}	0.008
Shear force (kg)	4.41±0.01 ^a	4.45±0.02 ^{ab}	4.46±0.03 ^{ab}	4.45±0.01 ^b	4.40±0.02 ^{ab}	0.012

¹100%DR is Duroc purebred, 50%DR x 50%LW is crossbred between 50%Duroc x 50%Large white, 50%DR x 50%LR is crossbred between 50%Duroc x 50% Landrace, 75%DR x 25%LW is crossbred between 50%Duroc and 25% Large white, and 50%DR x 25%LW x 25%LR is crossbred between 50%Duroc x 25%Large white x 25%Landrace. ²score typing of marbling; MB3 is three score marbling and MB6 is six score marbling.

Genetic parameters

Heritability, genetic parameters and phenotypic parameters for the traits are presented in Table 3. The estimates of heritability for marbling score and meat quality were moderate (0.19-0.38), except for color (L*), which has a high estimated heritability. These results confirm those of several previous studies, which reported the heritability of meat quality traits to range from 0.19-0.46 for color (Sonesson et al., 1998, Tholen et al., 2005) and highly heritable (0.45) for pork tenderness in both Duroc and Landrace pigs (Lo et al., 1992, Suzuki et al., 2005).

Table 3. Heritability (diagonal), genetic correlation (above diagonal) and phenotypic correlation (below diagonal) of marbling score and meat quality traits in purebred and crossbred Duroc pigs.

Traits ¹	MB3	MB6	Color (L*)	Cooking loss (%)	Shear Force
MB3	0.21	0.93	0.20	0.29	-0.43
MB6	0.94	0.19	0.05	0.29	-0.17
Color (L*)	0.01	-0.02	0.64	0.48	-0.52
Cooking loss (%)	-0.29	-0.33	0.26	0.38	-0.24
Shear Force (kg)	-0.74	-0.57	-0.06	0.08	0.22

¹score typing of marbling; MB3 is 3 score and MB6 is 6 score.

The phenotypic correlation was positive for marbling score (0.94) and negative for % cooking loss (MB3 vs % cooking loss = -0.29, MB6 vs % cooking loss = -0.33) and shear force (MB3 vs shear force = -0.74, MB6 vs shear force = -0.57). Genotypic correlation was positive with marbling score (0.93); thus a selection plan can be based on one trait for selection. Meanwhile, genetic correlation between marbling score and shear force was negative (MB3 vs shear force = -0.43, MB6 vs shear force = -0.17). As a result, improving marbling can lead to a decrease in shear force (more tenderness), which is consistent with previous reports (Ellis et al., 1996; Chen et al., 2014). For genotypic correlation between marbling score and % cooking loss was positive (0.29) in this study, which could be for several reasons, such as gene expression specific to the population, the pleiotropic effects of genes on multiple traits, or linkage disequilibrium among distinct loci (Falconer and

Mackay, 1996), which is in agreement with the results reported by Huff-Lonerger (2002). In addition, the genetic correlations between marbling score traits with color (L^*) were moderately positive. The color (L^*) was moderately correlated with % cooking loss, so the propensity to increase color (L^*) increases % cooking loss. The phenotype correlations between marbling score and color (L^*) and between tenderness and color (L^*) and cooking loss were quite low. Consequently, phenotypes with these traits were avoided.

PCR-RFLP genotyping and Genotype frequencies

Genotyping and gene frequencies are shown in Figure 2 and Table 4. All of the genes showed variation. However, genotype frequency, AA of the *CAST* gene and CC of *CAPNI* gene were detected at only 2 and 12%, respectively in this population, which is in contrast with the report of Gandolfi et al. (2011), who detected 10% AA genotype and zero for CC genotype in Italian Duroc×(Landrace×Large White) crossbred pigs.

Gene association

The association studies between candidate genes and meat quality traits are shown in Table 4 for both phenotype and genotype (EBV). The *ADRB3* variant c.1192G>A was found to be significantly associated with marbling score in both. An association between *ADRB3* gene polymorphism and porcine fatness traits was previously reported by Wang et al. (2013). It is noteworthy that the heterozygous genotype (AG) of the *ADRB3* gene has a higher marbling effect than the homozygous genotype (GG). It seems to be that the increased marbling of the AG genotype may be an over-dominance effect, which is consistent with a previous report (Wang et al., 2013).

In our study (Table 4), the *LIPE* c.442G>A SNP was not significantly associated with marbling score and meat quality traits in both phenotype and genotype (EBV). Indeed, the *LIPE* gene has an effect on accumulating fat in muscle and backfat content because it is involved with anti-lipolysis (Gerbens et al., 2000; Xue et al., 2015). Meanwhile, *LEPR* was not significantly associated for all meat quality traits as well. This result is in contrast with that of Lee et al. (2012), who reported that the *LEPR* gene has been used for a candidate gene to improve fatness traits. The *LEPR* gene has been associated with backfat thickness and intramuscular fat content (IMF) in Korean crossbred animals (Lee et al., 2012). The cause of non-significant SNPs in association studies may be due to different breeds, as Ovilo et al. (2005) found that the SNP at the same position in the *LEPR* gene was not significantly associated with IMF in an Iberian crossbred.

Calpain1 (*CAPNI*) g.157T>C was genotyped and used as a candidate gene because some studies have reported that calpain3 was not detected in pig meat (Parr et al., 1999; Gandolfi et al., 2011). In our study, *CAPNI* was significantly associated with the EBV of cooking loss percentage. The CC genotype showed a low cooking loss percentage. Indeed, *CAPNI* encodes the large subunit (μ -calpain) of an enzyme that has significant roles in proteolysis processes in pork (Huff-Lonerger and Lonergan, 2005; Kemp et al., 2010). High calpain activity leads to enhanced tenderness because it affects cleavage of specific myofibrillar proteins (Gandolfi et al., 2011). James and Yang (2011) reported that the water holding capacity of meat showed a strong linear correlation with tenderness. However, *CAPNI* was not significantly associated with shear force or tenderness/toughness in our

study. In contrast, porcine calpastatin (*CAST*) is an antagonist of calpain, which means that high activity leads to decreased proteolysis and enhanced meat toughness (Kent et al., 2004; Kemp et al., 2010). We found that the *CAST* gene was significantly associated with phenotypic and EBV shear force, with the GG genotype showing a lower shear force compared to the AG genotype. Moreover, *CAST* was associated with affected meat color, pH, water holding-capacity and texture parameters as well (Ropka-Molik et al., 2014).

Figure 2. Genetic polymorphisms of PCR products of candidate genes (*CAPN1*, *ADRB3*, *LEPR*, *CAST*, and *LIPE*) after amplifying with allele-specific primers and cutting with restriction enzymes: a) Calpain/HhaI: allele T (132, 65 bp) and allele C (91, 65, 41 bp); b) Adrenoceptor beta 3/TaqI: allele A (315 bp) and allele G (172, 143 bp); c) Leptin receptor/Tsp509I: allele A (184 bp) and allele G (113, 71 bp); d) Calpastatin gene/HinfI, allele A (376 bp) and allele G (129, 247 bp); and e) Lipase E hormone/BsaHI, allele A (307, 190 bp) allele G (240, 190, 67 bp) in pigs.

Table 4. Genotype frequency (in brackets) and candidate gene association analysis with phenotypic and estimated breeding value (EBV) of marbling score and meat quality traits in purebred and crossbred Duroc pigs.

Candidate genes ¹	Genotype	Phenotypic		Color (L*)	Cooking loss (%)	Shear Force	EBV		Color (L*)	Cooking loss (%)	Shear Force
		MB3	MB6				MB3	MB6			
	Frequency	(score ²)	(score ²)				(score)	(score)			
<i>LIPE</i> n=300	AA (0.11)	2.12	2.72	45.25	29.23	4.32	0.40	0.52	5.22	3.55	-0.003
	AG (0.46)	2.11	2.79	44.67	28.59	4.69	0.37	0.53	5.25	3.61	0.03
	GG (0.43)	2.14	2.70	44.61	28.74	4.39	0.39	0.47	5.25	3.46	0.00
	P-value	0.94	0.87	0.14	0.15	0.37	0.92	0.94	0.99	0.87	0.28
<i>LEPR</i> n=313	AA (0.19)	2.02	2.54	44.68	28.80	4.45	0.31	0.34	4.84	3.22	0.004
	AG (0.48)	2.12	2.75	44.97	29.01	4.54	0.37	0.50	5.42	3.61	0.024
	GG (0.33)	2.23	2.94	44.88	28.75	4.40	0.48	0.67	5.47	3.46	-0.004
	P-value	0.14	0.17	0.57	0.49	0.86	0.30	0.31	0.46	0.57	0.36
<i>ADRB3</i> n=305	AA (0.20)	2.16ab	2.80ab	44.91	29.05	4.37	0.39ab	0.53ab	5.35	3.66	-0.015
	AG (0.38)	2.22b	2.92b	44.70	28.68	4.34	0.48b	0.66b	5.29	3.38	0.015
	GG (0.42)	1.99a	2.49a	44.92	28.83	4.69	0.28a	0.32a	5.08	3.25	0.025
	P-value	0.01	0.03	0.55	0.42	0.37	0.05	0.04	0.83	0.58	0.28
<i>CAPN1</i> n=313	CC (0.12)	2.16	2.80	45.07	29.16	4.38	0.43	0.56	3.88a	2.64a	-0.01
	CT (0.35)	2.12	2.74	44.68	28.76	4.39	0.37	0.50	5.86b	3.83b	0.03
	TT (0.53)	2.10	2.67	44.77	28.64	4.62	0.36	0.45	5.97b	3.81b	0.00
	P-value	0.85	0.83	0.60	0.38	0.60	0.87	0.88	<0.01	0.03	0.19
<i>CAST</i> n=313	AA (0.02)	Non	Non	Non	Non	Non	Non	Non	Non	Non	Non
	AG (0.16)	2.11	2.66	44.66	28.53a	4.85a	0.35	0.41	4.98	3.09	0.05a
	GG (0.82)	2.15	2.81	45.03	29.17b	4.08b	0.42	0.60	5.51	3.78	-0.03b
	P-value	0.68	0.48	0.16	0.02	0.02	0.49	0.38	0.32	0.07	<0.01

¹ LIPE=Lipids E hormone, LEPR=leptin receptor, ADRB3=adrenoceptor beta 3, CAPN1=Calpain, CAST=calpastatin gene. ² Score 1= marbling score 1; Score 2= marbling score 2+3; Score 3= marbling score 4+5+6 Non = Non-analysis.

CONCLUSIONS

The heterozygous (AG) genotype of ADRB3 was significantly associated with marbling score. The CAPN and CAST genes were significantly associated with cooking loss and shear force, respectively. In breeding applications, the AG genotype of ADRB3 could be used as a marker for assisted selection to improve marbling scores. Also, the GG genotype of CAST is the most appropriate gene for selection of the pork tenderness trait.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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