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A single nucleotide polymorphism in CAST gene is associated with meat quality traits in rabbits*

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The purpose of this research was to investigate the effect of the Calpastatin (CAST) gene on meat quality traits in the Hyla, Champagne and Tianfu Black rabbit breeds. We detected one single nucleotide polymorphism (SNP: g.16443397 T>G) located at 271bp in intron 8 in the Hyla, Champagne and Tianfu Black rabbit breeds. This SNP was significantly related to pH and intramuscular fat in rabbit meat from the *longissimus dorsi* and *biceps femoris* muscles. Rabbits with the GG and TT genotypes had lower pH and intramuscular fat content than GT rabbits. A comparison of meat quality trait least squares means for the *longissimus dorsi* and *biceps femoris* muscles indicated that pH values were similar in both *longissimus dorsi* and *biceps femoris* muscles, color lightness in the *biceps femoris* muscle was higher than that in *m. longissimus dorsi*, while color redness and yellowness in *m. longissimus dorsi* were higher than in the *biceps femoris* muscle. Thus, results reported here indicated that this CAST SNP may be of potential use in marker assisted selection for meat quality traits in rabbits.

KEYWORDS: rabbit/ CAST/ SNP/ meat quality

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Calpastatin (CAST) is found commonly in muscle cells and was first identified in 1978. Calpastatins are rich in proline and glutamate, but poor in aromatic amino acids [Murachi 1983]. Calpastatin is also an endogenous inhibitor that controls the activity of calpains in the presence of Ca^{2+} [Kwak *et al*. 1993]. The calpain-calpastatin enzyme complex regulates the rate of protein degradation in the live animal [Koohmaraie *et al.* 2002]. Calpain activity accelerates protein degradation, whereas calpastatin acts as an inhibitor. The rate of protein degradation post mortem affects meat quality [Koohmaraie *et al*. 2002]. Variation in calpastatin abundance influences postmortem aging rates in different muscles [Ouali *et al*. 1990, Geesink *et al.* 1992]. Recently, several researches showed that single nucleotide polymorphisms in the calpastatin gene were associated with beef tenderness [Calvo *et al*. 2014, Li *et al.* 2013]. Other reports showed that the CAST gene has the greatest effect on pork quality [Ropka-Molika *et al*. 2014, Rohrer *et al*. 2012] and muscle fiber traits in chickens [Liu *et al*. 2008, Zhang *et al*. 2012]. However, no papers were found on the association between SNPs and rabbit meat quality traits. Thus, the objective of this study was to investigate single nucleotide polymorphisms in the CAST gene and to test their association with meat quality traits in Hyla, Champagne and Tianfu Black rabbit breeds.

Material and methods

This study was carried out at the experimental rabbitry of the Institute of Animal Genetics and Breeding, Sichuan Agricultural University, China. A total of 361 rabbits were used, including 129 Hyla (HY), 141 Champagne (AC) and 91 Tianfu Black (TB). Rabbits were reared in individual cages after being weaned at 6 weeks and they were fed ad libitum with commercial diet. Rabbits were slaughtered at 70 days of age and samples of ear tissue were collected for DNA extraction. Carcasses were kept at 4° for 24 hours. Meat quality measurements were: 1) pH at 0 h and 24 h post morterm, 2) color at 0 h and 24 h post morterm, 3) cooking loss at 24 h post morterm, and 4) intramuscular fat (IMF) at 24 h post mortem. Measurements of pH, IMF, color and IMF were taken from the *biceps femoris* and *longissimus dorsi* muscles, whereas cooking loss was measured only in the *biceps femoris* muscle.

Genomic DNA was extracted using the AxyPrep Genomic DNA Miniprep Kit (Axygen, USA) and stored at -20°. The PCR primers for the CAST gene were designed by the Primer Premier 5 software based on the rabbit gene sequence (Ensembl accession NO. ENSOCUG00000007802). The PCR primers for the CAST gene were as follows: CAST-F: AAACTTGACAGCCACCTGTGGAT, CAST-R: CCGTATGGAATTGGCTGGTTG. These PCR primers were used to amplify a 659 bp fragment of the CAST gene that included exon 8 5, intron 8 5 and exon 9 6. The 25μL reaction mixture contained 50 ng genomic DNA, 1 uM of each primers, 1.5 mM $MgCl_2$, 200 μM dNTPs (dATP, dTTP, dCTP and dGTP), and 0.4 units of Taq DNA polymerase (MBI). The PCR protocol involved initial denaturation at 95° for 5 min, 35 cycles of denaturation at 95° for 45 s, annealing at 56.5° for 45 s, extension at 72° for 45 s, with final extension at 72° for 10 min. Then, the PCR products were

directly sequenced on a 3700 DNA sequencer in both directions using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Next, the sequences were analyzed with DNAMAN software (version 5.2.2).

The genotype and allele frequencies in all breeds were calculated. The Hardy-Weinberg equilibrium (HWE) was tested for different locus-population combinations and a number of observed and effective alleles using the likelihood ratio test with the POPGENE software (Ver. 3.2) [Yeh *et al.* 1997]. The population genetic indexes including expected gene heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) were estimated using the following formulas [Nei *et al.* 1974, Botstein *et al*. 1980]:

$$He = 1 - \sum_{i=1}^{n} P_i^2 ,$$

$$Ne = 1 / \sum_{i=1}^{n} P_i^2 \text{ , and}$$

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where P_i was the frequency of the *i*th allele and *n* was the number of alleles.

Considering time of measurement (0 h and 24 h post mortem) and muscle sample (*biceps femoris* and *longissimus dorsi*), there were a total of 19 meat quality traits: pH, color lightness, color redness, and color yellowness taken at 0 h and 24 h post mortem in the *biceps femoris* and *longissimus dorsi* muscles, cooking loss at 24 h post mortem in the *biceps femoris* only, and IMF at 24 h post mortem in the *biceps femoris* and *longissimus dorsi* muscles. Meat quality traits were analyzed using the following linear model:

$$Y_{ijkl} = \mu + B_i + G_j + S_k + e_{ijkl}$$

where Y_{ijkl} was a meat quality trait, μ was the overall mean, B_i was the breed effect, G_j was the genotype effect, S_k was the fixed sex effect, and e_{ijkl} was the random error. Least squares means and their standard errors were computed and pairwise comparisons among them were made using Bonferroni t-tests. Computations were carried out using the general linear model (GLM) procedure of SPSS 21 (IBM, Armonk, NY, USA).

Results and discussion

SNP identified

We detected one single nucleotide polymorphism located at 271 bp in intron 8, while no SNPs were found in exons 8 and 9 from the set of animals used in this study. SNP genotyping was successfully implemented using DNA sequencing and polymerase chain reaction (PCR). Figure 1 shows a sequencing map of the novel

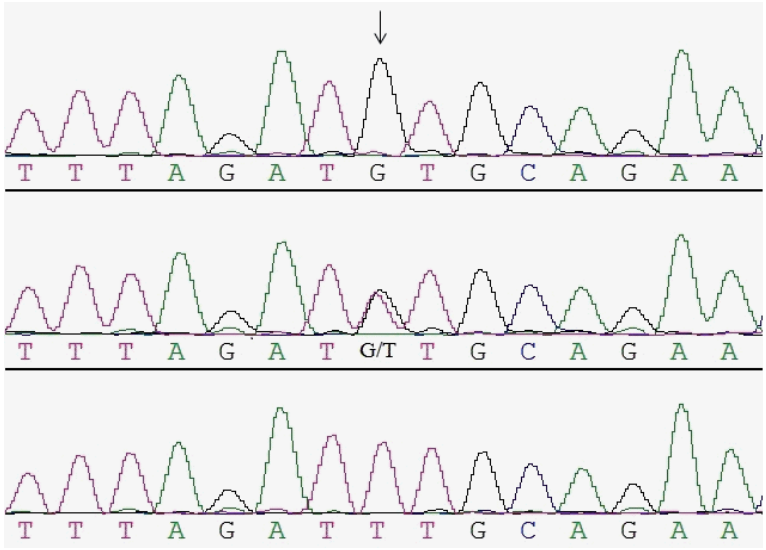

Fig. 1 Sequencing map of three genotypes of the rabbit CAST gene in the intron 8 region.

Table 1 Genotypic and allelic frequencies for the rabbit CAST gene

Breed	No. rabbits	CAST genotype number and frequency[1]			CAST Allele frequency	
		GG	*TT*	*GT*	*G*	*T*
Hyla	129	38 (0.295)	70 (0.543)	21 (0.163)	0.376	0.624
Champagne	141	21 (0.149)	82 (0.582)	38 (0.270)	0.284	0.716
Tianfu Black	91	16 (0.176)	53 (0.582)	22 (0.242)	0.297	0.703
Total	361	75 (0.208)	205(0.568)	81 (0.224)	0.320	0.680

[1]In parentheses.

SNP-T>G (g.16443397, in Chromosome 11) of rabbits. Three genotypes (GG, TT and GT) were identified, counted, and the genotype and allele frequencies were calculated in the Hyla, Champagne and Tianfu Black breeds (Tab. 1). The genotype frequency of TT was much higher than GG and GT. The T allele frequency (0.6240 to 0.7163) was also much greater than that of G, which showed a high prevalence in these breeds. The minor allele frequencies (MAF) ranged from 0.2837 to 0.3760. These allele frequencies showed that this SNP was polymorphic (MAF>0.05) in these three breeds. Chi-square tests showed that genotypic frequencies were not in Hardy-Weinberg equilibrium ($P<0.05$) in any of the three rabbit populations, which may have been due to the limited sample size in these populations and artificial selection of parents for traits associated with the meat quality traits considered here (e.g. carcass weight, visual meat appraisal, meat taste).

Diversity analysis

The values of three population genetic indices (H_o, H_e, and N_e) are presented in Table 2 to evaluate diversity of the three rabbit breeds. All values of H_e were above 0.4 and below 0.5, whereas values of N_e were close to 0.2. In order to measure the informativeness of the identified SNP, the PIC value was calculated for each breed of rabbit. Based on the classification of PIC (PIC value <0.25, low polymorphism; PIC value >0.50, high polymorphism; 0.25<PIC value <0.50, intermediate polymorphism), the three rabbit populations had intermediate levels of genetic diversity, which indicated that the rabbit population exhibited sufficient diversity for selection of potential and improvement of production to be effective in each of the three rabbit populations for the meat quality traits analyzed in this study.

Table 2 Chi-square test values and diversity parameters for the rabbit CAST gene

Breed	No. rabbits	χ^2 (HWE)[1]	He[2]	Ne[3]	PIC[4]
Hyla	129	55.0186*	0.4692	1.8841	0.3591
Champagne	141	16.0019*	0.4064	1.6847	0.3238
Tianfu Black	91	16.1073*	0.4173	1.7163	0.3303
Total	361	84.6997*	0.4351	1.7703	0.3405

[1]χ2 (HWE) – Hardy-Weinberg equilibrium χ2 value. [2]He – gene heterozygosity.
[3]Ne – effective allele number. [4]PIC – polymorphism information content.
*P<0.05.

Association analysis

The results of the association analysis between SNP and rabbit meat quality are shown in Tables 3, 4 and 5. Table 3 shows least squares means of L* (0h, 24h), a* (0h, 24h), b* (0h, 24h) for CAST genotypes GG, TT, and GT in the *longissimus dorsi* muscle whereas Table 4 presents least squares means for these same traits and genotypes for the *biceps femoris* muscle. Table 5 contains least squares means for GG, TT, and GT for cooking loss (combined samples of *longissimus dorsi* and *biceps femoris* muscles) and intramuscular fat content for *longissimus dorsi* muscle samples only and for *biceps femoris* muscle samples only.

Least squares means in Table 3 indicated that rabbits with the GT genotype had significantly greater pH_{0h} and b^*_{0h} than those with the TT genotype, but similar values for pH_{24h}, L* (0h, 24h), a* (0h, 24h), and b^*_{24h} in the *longissimus dorsi* muscle. Similarly, rabbits with the GT genotype had significantly greater pH_{0h} and b^*_{0h} than those of TT rabbits for the *biceps femoris* muscle. In addition, GT rabbits had significantly higher pH_{24h} than rabbits with the GG genotype for the *biceps femoris* muscle, while similarly as with the longissimus dorsi muscle, non-significant differences were recorded between the 3 genotypes for L* (0h, 24h), a* (0h, 24h), and b^*_{24h}. Thus, the pH value of meat stored 24 h was lower than the pH value of fresh meat, but it was significantly different only for the *longissimus dorsi* muscle. Lastly, the values of L* and b* increased and the values of a* decreased in the *longissimus dorsi* and *biceps femoris* muscles after 24 h for all the 3 genotypes (GG, TT, and GT).

Table 3. Least square means for CAST genotype effects on meat pH and color traits in rabbit *longissimus dorsi* muscle

Trait	CAST Genotype			*P*-value
	GG	*TT*	*GT*	
pH_{0h}	6.71 (0.08)^{ab}	6.36 (0.08)^{b}	6.92^{a} (0.03)	**0.047**
pH_{24h}	5.73 (0.04)	5.76 (0.03)	5.69 (0.01)	0.184
L^*_{0h}	47.53 (0.91)	49.67 (0.90)	52.26 (1.0)	0.175
L^*_{24h}	58.99 (0.96)	59.10 (0.59)	59.32 (0.77)	0.772
a^*_{0h}	4.84 (0.45)	5.17 (0.32)	4.99 (0.26)	0.383
a^*_{24h}	4.46 (0.49)	4.52 (0.45)	4.11 (0.49)	0.733
b^*_{0h}	2.68 (0.21)^{ab}	2.15 (0.17)^{b}	3.62 (0.31)^{a}	**0.038**
b^*_{24h}	5.72 (0.27)	4.73 (0.27)	4.28 (0.23)	0.326

^{ab}Means bearing different superscripts differ significantly at P<0.05.
L^* – Lightness from 0 (black) to 100 (white); a^* – redness from -60 (green) to 60 (red); b^* – yellowness from -60 (blue) to 60 (yellow).

A comparison of meat quality trait least squares means for the *longissimus dorsi* muscle (Tab. 3) and *biceps femoris* muscle (Tab. 4) indicated that: 1) L* values in the *biceps femoris* muscle were higher than that in *longissimus dorsi*; 2) a* and b* values in *longissimus dorsi* were higher than in the *biceps femoris* muscle; and 3) pH values were similar in both *longissimus dorsi* and *biceps femoris* muscles.

Table 4. Least square means for CAST genotype effects on meat pH and color traits in rabbit *biceps femoris* muscle

Trait	CAST Genotype			*P*-value
	GG	*TT*	*GT*	
pH_{0h}	6.63 (0.11)^{ab}	6.39 (0.26)^{b}	6.82 (0.10)^{a}	**0.039**
pH_{24h}	5.81 (0.02)^{a}	5.79 (0.03)^{ab}	5.62 (0.04)^{b}	**0.025**
L^*_{0h}	53.57 (1.0)	52.63 (0.61)	51.10 (0.47)	0.229
L^*_{24h}	61.36 (1.01)	63.55 (0.92)	62.73 (0.62)	0.508
a^*_{0h}	4.39 (0.45)	3.89 (0.30)	4.62 (0.44)	0.358
a^*_{24h}	4.10 (0.45)	3.60 (0.38)	4.03 (0.48)	0.267
b^*_{0h}	1.81 (0.23)^{ab}	1.07 (0.19)^{b}	2.46 (0.25)^{a}	**0.039**
b^*_{24h}	4.77 (0.34)	5.41 (0.41)	5.67 (0.42)	0.490

^{ab}Means bearing different superscripts differ significantly at P<0.05.
L^* – Lightness from 0 (black) to 100 (white); a^* – redness from -60 (green) to 60 (red); b^* – yellowness from -60 (blue) to 60 (yellow).

Our study indicated that pH in fresh rabbit meat was higher than that in meat stored for 24h. Zeferino *et al*. [2013] found no difference in pH values in rabbit meat stored for 24 h and 48 h. Higher pH levels are probably associated with a greater depletion of muscle glycogen reserves [Sabuncuoglu *et al*. 2011] and may favor the development of proteolytic microorganisms [Dalle Zotte 2002]. Color values were similar to values reported in previous research [María *et al*. 2006, Trocino *et al*. 2002]. The small differences in color trait values between *longissimus dorsi* and *biceps femoris* muscles here partially agreed with the results reported by Chiericato *et al.* [1996b], who found that yellowness was

not consistent in different muscles. Conversely, long-term artificial selection for growth rate was found to have some effect on myofiber metabolism [Bianospino et al. 2008], which further influenced meat color in agreement with previous research, in which lower yellowness values were observed in rabbits selected for growth rate when compared to the control group [Ramírez *et al*. 2004, Pascual *et al*. 2007].

Table 5. Least square means for CAST genotype effects on meat cooking loss and intramuscular fat in rabbit

Trait	CAST Genotype			*P*-value
	GG	*TT*	*GT*	
Cooking loss (%)[1]	35.28 (1.29)	32.05 (1.75)	29.96 (1.45)	0.376
IMFLD[2]	0.201 (0.002)[a]	0.98 (0.023)[b]	0.018 (0.002)[c]	0.028
IMFHL[3]	1.002 (0.067)[a]	0.86 (0.034)[a]	0.023 (0.003)[b]	0.032

[1]*Biceps femoris* muscles only. [2]*Longissimus dorsi* muscle only. [3]*Biceps femoris* muscle only.
[abc]Means bearing different superscripts differ significantly at P<0.05.

Cooking loss was evaluated using all muscle samples (i.e., *longissimus dorsi* and *biceps femoris*). Although the least squares mean for GT rabbits was lower than those for GG and TT rabbits, no significant differences existed among the GG, TT, and GT genotypes (Tab. 5). Rabbits with the GT genotype had a significantly lower intramuscular fat content in the *longissimus dorsi* and *biceps femoris* muscles than those with genotypes TT and GG. Intramuscular fat content was significantly higher in rabbits with the TT genotype in *longissimus dorsi*. However, no significant differences existed between GG and TT rabbits for intramuscular fat in the *biceps femoris* muscle, although the least squares means for GG rabbits were somewhat higher than that for TT rabbits. Compared to pig meat, rabbit meat has a lower intramuscular fat and higher cooking loss [Lim *et al*. 2014]. Lower intramuscular fat and higher cooking loss is unfavorably related to eating quality for most consumers and affects tenderness, flavor, and fat firmness [Lim *et al*. 2014, Wood *et al.* 2003]. However, lower intramuscular fat is good for human health. This would suggest that the lower cooking loss and intramuscular fat values for the GT genotype would be more beneficial to human health than the higher values for the GG and TT genotypes.

It could be concluded that this CAST SNP is of potential use in marker assisted selection for meat quality traits in rabbits. It assumes that this SNP is closely associated with QTL affecting meat quality traits. Further work is necessary in different, larger rabbit populations to elucidate the mechanisms involved in the effect of this gene on rabbit meat quality.

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