New alleles in calpastatin gene are associated with meat quality traits in pigs[1]

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ABSTRACT: Suggestive QTL affecting raw firmness scores and average Instron force, tenderness, juiciness, and chewiness on cooked meat were mapped to pig chromosome 2 using a three-generation intercross between Berkshire and Yorkshire pigs. Based on its function and location, the calpastatin (*CAST*) gene was considered to be a good candidate for the observed effects. Several missense and silent mutations were identified in *CAST* and haplotypes covering most of the coding region were constructed and used for association analyses with meat quality traits. Results demonstrated that one *CAST* haplotype was significantly associated with lower Instron force and cooking loss and higher juiciness and, therefore, this haplotype is associated with higher eating quality. Some of the sequence variation identified may be associated with differences in phosphorylation of *CAST* by adenosine cyclic 3′, 5′-monophosphate-dependent protein kinase and may in turn explain the meat quality phenotypic differences. The beneficial haplotype was present in all the commercial breeds tested and may provide significant improvements for the pig industry and consumers because it can be used in marker-assisted selection to produce naturally tender and juicy pork without additional processing steps.

Key Words: Calpastatin, Haplotype, Meat Quality, Pig, Tenderness

Introduction

Eating quality of meat depends on several important characteristics, including appearance, color, taste, fat content, texture, and tenderness. Meat quality is affected by postmortem tenderization, which is a complex structural and functional process that depends on species, genetic background, metabolic status of the animal antemortem, the protein complement of the muscle, and environmental factors. The rate of postmortem proteolysis of several important structural muscle proteins is considered a determinant factor in postmortem tenderization (Taylor et al., 1995).

Calpastatin (**CAST**) is a specific inhibitor of μ- and m-calpain (EC 3.4.22.17) proteases. There is evidence indicating that in different species, calpastatin activity postmortem is highly related to meat tenderness (Koohmaraie et al., 1991; Sensky et al., 1998; Parr et al., 1999).

Starting with Lonergan et al. (1995), several association studies have investigated sequence variation in calpastatin as a source of genetic markers that may influence meat tenderness. Although some suggestive associations were reported in cattle (Chung et al., 1999, 2002; Barendse, 2002) and pigs (Kocwin-Podsiadla et al., 2003), none of these studies found any AA sequence variation or changes in the potential regulatory regions that could explain phenotypic differences in meat quality.

Using a Berkshire × Yorkshire pig family (Malek et al., 2001b), suggestive QTL for raw firmness score, cooked meat quality traits, including average Instron force, and the sensory traits juiciness, chewiness, and tenderness scores were revealed on swine chromosome 2 (**SSC2**). Based on its function and location, *CAST* was considered to be a good candidate gene for these QTL. The objective of this study was to evaluate this posi-

tional candidate gene for association with variation in pork quality.

Materials and Methods

Tissue Sampling and DNA/RNA Isolation

Blood samples and phenotypes were collected and recorded on the F_0, F_1, and F_2 animals (n = 570) from the intercross of a Berkshire × Yorkshire (**B × Y**) three-generation family (Malek et al., 2001b), as well as blood samples and muscle tissue from the ham and loin muscles from more than 10 individual F_3 animals for RNA/DNA isolation purposes. A large set of tissue samples from four different commercial purebred lines of pigs were collected at a single plant over a 3.5-yr period before 2001. These animals were produced at two different farms and slaughtered over 73 slaughter dates. The lines of pigs included Large White, Duroc, Duroc/Large White Synthetic, and a Composite line. Several of these lines were used to produce a set of crossbred slaughter pigs. Tissue samples were collected for RNA/DNA isolation purposes from the longissimus dorsi of eight Large White individuals and one Meishan. Genomic DNA was isolated either from whole blood by standard salting out procedures or from ear notches using the DNeasy tissue kit (Qiagen, Valencia, CA). Total RNA was extracted from ham (semimembranosus) and loin muscle tissue (longissimus dorsi) using the TRIzol reagent method according to manufacturer instructions (Invitrogen-Life Technologies, Carlsbad, CA).

Phenotypic Trait Measurement

$B × Y$ F_2 Reference Family. Meat quality measures were made on the longissimus dorsi and included firmness on raw meat, several sensory traits (juiciness, tenderness, and chewiness score) on cooked meat, and average Instron force. For trait descriptions and methods used, see Malek et al. (2001a,b).

Commercial Purebred Pig Lines. Subjective firmness was measured and scored from 1 to 5 on the longissimus dorsi, with higher values indicating greater firmness. Scores were determined visually on raw meat at a packing plant 24 h after slaughter on individuals sampled from the four purebred commercial pig lines (Berg, 2000).

Commercial Crossbred Slaughter Pigs. Longissimus dorsi pH, instrumental tenderness (shear force), cooking loss, and sensory measurements (juiciness and tenderness) were evaluated at the University of Illinois after 14 d aging. Shear force was measured with an Instron 1222 Universal Testing Machine (Instron, Canton, MS) fitted with a Warner-Bratzler shear attachment. Sensory juiciness and tenderness were scored from 1 to 10, with higher values indicating juicier or more tender meat. For cooking loss measurements, a chop was cut from the longissimus dorsi muscle after aging, weighed, cooked to 70°C in a Farberware open-hearth electric grill (Farberware Inc., Westbury, NY), refrigerated until cool, and reweighed. During cooking, the temperature in each chop was monitored in its center with thermocouples. Cooking loss was calculated from weights taken before and after cooking and was expressed as a percent.

Linkage Mapping and QTL Analysis

An intercross between B × Y pig breeds, yielding 525 F_2 offspring, was generated to map QTL for meat quality, growth, and carcass composition (Malek et al., 2001a,b). Additional microsatellite markers were subsequently genotyped across the three-generation family to increase the number of informative meioses in the region of the *CAST* gene, which seemed to harbor QTL for firmness and related traits (Thomsen et al., 2002). Mapping of the *CAST* gene to the SSC2 linkage map was performed by using a previously reported *CAST MspI* substitution (Ernst et al., 1998), and was accomplished using CRI-MAP (Green et al., 1990).

PCR, RT-PCR, and Polymorphism Discovery

Based on the *CAST* pig cDNA sequence available in GenBank (M20160), primers were designed to amplify the entire coding region of the type III *CAST* skeletal muscle isoform. The cDNA obtained from B × Y F_3 individuals and from Large White and Meishan individuals were used in polymorphism discovery. The PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and standard PCR protocols. Reverse transcription of total RNA was performed by random hexanucleotide priming and Superscript II (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol, and PCR primers were designed to amplify the full coding region of the type III *CAST* isoform (primers: set A, forward 5′ CCGAGCCC AACCAGGAATGC 3′ and reverse 5′ TTTGTCCTTGA CCTCTTCTAATC 3′; set B forward 5′ CCAGAAGATG GAAAGCCTGTG 3′ and reverse 5′ TGAAGATTGTCT ATGCAGATACC 3′). Amplicons were sequenced using dye terminators on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The Sequencher software (Gene Codes, Ann Arbor, MI) was used to assemble the sequences and to identify polymorphisms. The sequence polymorphisms were confirmed using restriction enzyme tests.

Genotyping and PCR-RFLP Analysis

The region flanking each newly discovered missense mutation (*Ser66Asn*, *Arg249Lys*, and *Ser638Arg*) was PCR amplified (primers: *Ser66Asn* forward 5′ GTAAA GCCAAAGGAACACCCAG 3′ and reverse 5′ TTTTTA TTTCTCTGATGTTGGCTGTGCA 3′; *Arg249Lys*, forward 5′ AAATCTACTGGAGAGGTTTTGAA 3′, and reverse GACTTCTCCCGAATCAGTTCC; *Ser638Arg*, forward 5′ AAACCTATTTTCAGGGATATGGG 3′ and reverse 5′ CCTTTGTTGTGTTCTCTGAGG 3′) and then

digested with *Apa*LI (*Ser66Asn*), *Hpy*188I (*Arg249Lys*), and *Pvu*II (*Ser638Arg*). The *Ser66Asn* reverse primer was modified relative to the original swine cDNA sequence to create an *Apa*LI restriction polymorphism to be able to differentiate the alleles by PCR-RFLP. The *Ser66Asn* substitution was not used in these association studies because initial analysis with 200 individuals from eight Western breeds revealed that it was in complete linkage disequilibrium with *Arg249Lys*. Potential phosphorylation sites for adenosine cyclic 3′, 5′-monophosphate dependent protein kinase (**PKA**) were identified using NetPhos 2.0 prediction server (Blom et al., 1999).

Statistical Analyses

Berkshire × Yorkshire F$_2$ Population Analysis. Haplotypes defined by the *CAST Arg249Lys* and *CAST Ser638Arg* polymorphisms were assigned in the B × Y population based on complete genotyping of the parents. The substitution effects of the haplotypes were estimated using a mixed model (SAS PROC MIXED, SAS Inst., Inc., Cary, NC) that included litter as a random effect, and slaughter date and sex as fixed effects. One variable was included for each haplotype, with values –1, 0, and 1 corresponding to the animal having 0, 1, or 2 copies of the haplotype in question. The effect of the haplotype variables was tested by calculating the likelihood ratio of this full model and a reduced model without the haplotype variables. Significance *P*-values were obtained from a χ^2 distribution with 2 df for the values of –2 times the log likelihood ratio. Haplotype substitution effects were estimated for traits affected (*P* < 0.10 or *P* < 0.20 in more then one independent data set) by the *CAST* haplotypes.

Commercial Purebred Lines Analyses. Haplotypes defined by the *CAST Arg249Lys* and *CAST Ser638Arg* polymorphisms were predicted based on inferences derived from the genotypes of homozygous individuals. Haplotype analysis was performed (as described in the previous paragraph) on the combined data from four commercial lines with a mixed model (SAS PROC MIXED) that included sire (n = 165) as a random effect. Potential relationships between the sires were ignored in the analysis. Slaughter date and line were included as fixed effects. Sex and farm were not included because all traits were measured only in females and only one farm was represented on each slaughter date. Associations between the individual *CAST* polymorphisms *CAST Arg249Lys* and *Ser638Arg* and meat quality traits also were tested by replacing the haplotype variables with marker genotype as a fixed effect (*P* < 0.10). Line × genotype interactions were tested in this model and not found to be significant. Least Squares (**LS**) means for the three genotype classes were obtained for both polymorphisms and contrasts tested for significance (nominal *P* < 0.05).

Commercial Crossbred Pig Data Analysis. No pedigree information was available for this data set, but haplotypes were predicted based on inferences derived from the genotypes of homozygous individuals. Analysis of haplotype associations was based on a model with the breed composition of the six crossbred products included as a fixed effect and haplotypes as described under the statistical analysis for the B × Y F$_2$ population.

Results

Linkage Mapping and Marker Development

In the B × Y intercross family, suggestive QTL were detected on SSC2 in the area where the calpastatin gene was expected to be located for several traits including firmness on raw meat and average Instron force, sensory juiciness, tenderness, and chewiness score (Malek et al., 2001b). Using additional markers and a previously reported *CAST* polymorphism (*CAST – Msp*I; Ernst et al., 1998), a new SSC2 map was developed. Extended QTL analysis of this B × Y family for growth and meat quality traits (Thomsen et al., 2002) using the new map confirmed a QTL significant at the 5% chromosome-wise level for firmness (Figure 1; Table 1), and a nominally significant QTL for average Instron force and juiciness in the *CAST* region (Figure 1; Table 1). The firmness QTL was located approximately 13 cM away from the main group of QTL. The favorable alleles at these QTL each had an additive effect and were derived from the Berkshire breed, which is generally regarded as having very good meat quality (Table 1).

Based on its function and map location (Rattink et al., 2001a,b), the *CAST* gene was a good candidate gene in this chromosomal region. By sequencing the entire coding region of the *CAST* gene, including parts of 5′ and 3′ untranslated areas, in B × Y F$_3$ individuals with extreme values for meat quality, three missense mutations (*CAST Ser66Asn* located in Domain L, *CAST Arg249Lys* in Domain 1, and *CAST Ser638Arg* in Domain 4) were identified (see Figure 2). In the B × Y family and also in 200 individuals from several commercial lines, the *CAST Ser66Asn* polymorphism was in complete linkage disequilibrium with the *CAST Arg249Lys* polymorphism. Five silent mutations were also found in the B × Y resource family. Additional sequencing using several individuals from a Large White line and a Meishan individual revealed four additional polymorphisms. These included one in the 5′ untranslated region (**UTR**), a *Leu306Val* missense mutation located in Domain 2 (in a nonconservative position within Subdomain B), and two Meishan specific mutations: one silent and one missense, the last one being located in Domain 3, Subdomain C (Figure 2). Differences in allele frequency at each polymorphic site between the founders of the intercross family were intermediate (e.g., 0.75 and 0.39 for the *Arg249Lys* and 0.75 and 0.50 for the *Ser638Arg* in the Berkshire and Yorkshire founders, respectively).

Haplotypes covering most of the gene were constructed using all of the polymorphisms that were iden-
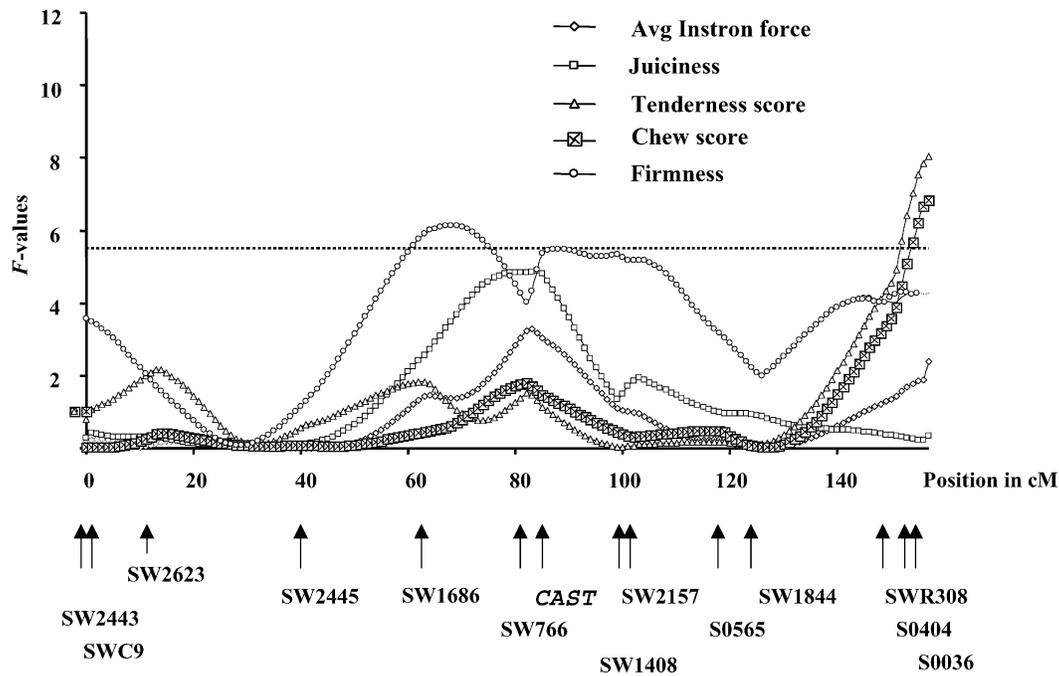
Figure 1. The QTL scan *F*-ratio curves for evidence of QTL associated with meat quality traits for chromosome 2. The x-axis indicates the relative position on the linkage map. The y-axis represents the *F*-ratio. Arrows on the x-axis indicate the position where a marker is present. Dotted line represents the 5% chromosome-wide significance.

tified by sequencing *CAST* cDNA from more than 20 individuals belonging to B × Y F₃, Large White, and Meishan breeds (Figure 2). Four haplotypes were determined (B × Y and sequencing work) and/or predicted to be present in the populations used here, three were found at a significant frequency in most of the Western type commercial breeds (Haplotypes 1, 2 and 3), and the fourth was Meishan specific (Haplotype 4). Although additional sequencing may reveal more haplotypes, their frequency is expected to be low so that they would not be useful for analysis of this data set.

Association Study

Berkshire × Yorkshire F₂ Population Analysis. Two of the polymorphisms, *Arg249Lys* and *Ser638Arg*, were

considered to be the most interesting because of their missense characteristics and because these two polymorphisms, assembled together in haplotypes, specified the three individual haplotypes revealed in the *CAST* gene in the commercial populations (Figure 2). These three haplotypes (1, 2, and 3) were also present in the founders of the B × Y resource family. As with the individual substitution sites, the differences in haplotype frequency were not large between the founders of the intercross family (e.g., 0.75 and 0.39 for Haplotype 1, 0 and 0.11 for Haplotype 2, and 0.25 and 0.5 for Haplotype 3 in the Berkshire and Yorkshire founders, respectively).

Individual substitution effects were analyzed and significant differences were detected for both polymor-

Table 1. Evidence for suggestive quantitative trait locus on chromosome 2 for various meat quality traits in the Berkshire-Yorkshire F₂ population

Trait	*F*-value[a]	Location, cM	Additive Effect[b]	SE	Dominance Effect	SE	Variance, %[c]
Firmness score	6.14	68	−0.14	0.04	−0.005	0.06	2.95
Avg Instron force, kg	3.27	83	−0.14	0.06	0.01	0.08	1.75
Sensory tenderness score	1.52	82	0.14	0.08	0.01	0.12	0.75
Sensory chewiness score	1.79	82	−0.11	0.06	−0.05	0.09	0.91
Sensory juiciness score	4.89	84	0.30	0.10	0.04	0.14	2.50

[a]Chromosome-wise *F*-statistic threshold at the 5% level, as determined by permutation test was 5.5.
[b]Additive (a) and dominance (d) QTL effects correspond to genotype values of +a, d, and −a , respectively, for individuals having inherited two Berkshire alleles, heterozygotes, and individuals with two Yorkshire alleles. Positive additive effects indicate that Berkshire alleles increased the trait, negative that the Berkshire alleles decreased it. Dominance effects are relative to the mean of the two homozygotes.
[c]Variance, % = variance at the QTL based on estimated additive and dominance effects and allele frequencies of 1/2, as a percentage of the residual variance in the F₂.
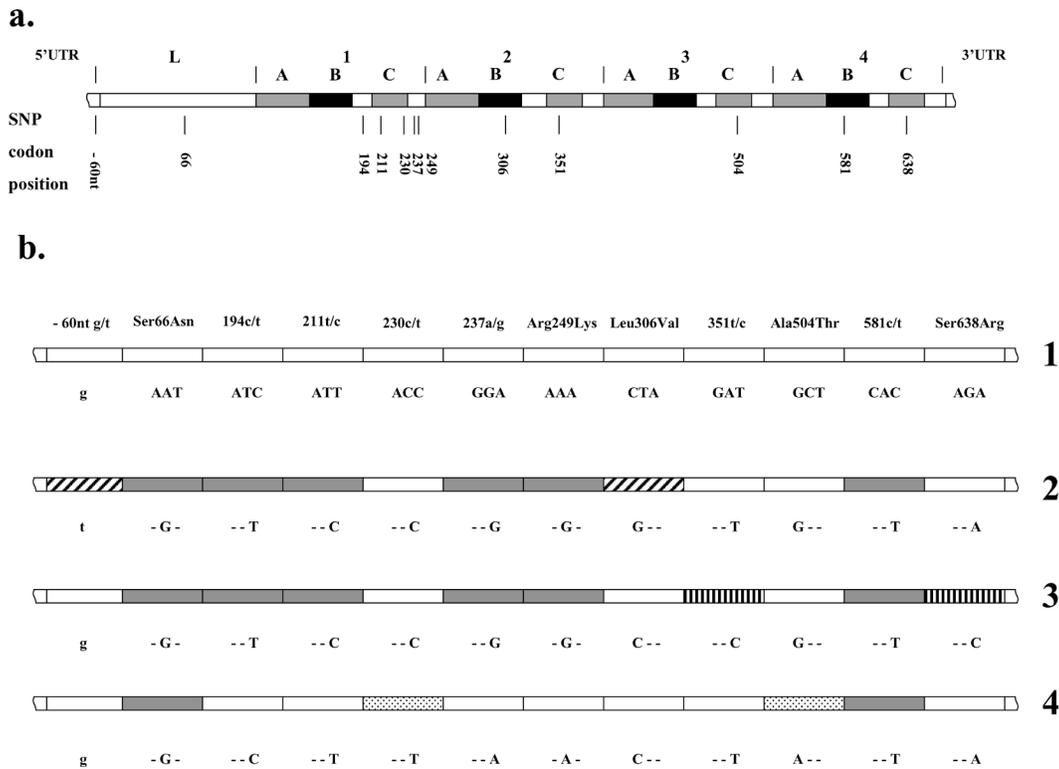
a.

b.

Figure 2. The location of substitutions in calpastatin (*CAST*) (a) and the haplotypes constructed using the sequence variation (b). The positions of the coding polymorphisms are presented as codon positions. The substitution from the 5′untranslated (5′UTR) is presented as a nucleotide (nt) position relative to the proposed translation start site for the type III CAST isoform.

phisms (results not shown); however, because of the significant linkage disequilibrium between the *CAST* polymorphisms, the haplotypes were tested instead of the individual polymorphisms and found to have an effect on firmness ($P < 0.01$), Instron force ($P < 0.01$), juiciness score ($P < 0.05$), and a trend was seen for chewiness score ($P < 0.10$). Effects were estimated as contrasts between the variables representing the observed haplotypes (Table 2). Substitution effects were discovered in raw muscle for firmness between Haplotypes 1 and 2 ($P < 0.01$) and between Haplotypes 2 and 3 ($P < 0.05$). A substitution effect was also revealed for meat quality in cooked meat between Haplotypes 1 and 3 for subjective juiciness ($P < 0.01$), chewiness ($P < 0.05$), and average Instron force ($P < 0.01$). Tenderness score was not significant but haplotype substitution effects were included in Table 2 because the direction of the substitution effects were the same as for the other sensory traits and because the same trait was found at $P < 0.20$ in this data set as well as in the commercial crossbred pig data set (see Table 5). Haplotype 1 was the favorable haplotype for all traits measured on cooked meat, as it is associated with higher juiciness and lower chewiness and average Instron force. Haplotypes 1 and 3 were associated with a softer muscle compared with Haplotype 2.

Commercial Purebred Lines Analyses. Of the traits found to be significant in the B × Y population only firmness scores were available to evaluate the potential roles of *CAST* haplotypes defined by *Arg249Lys* and *Ser638Arg*, across the four commercial lines. A line × genotype interaction was not detected for individual substitution analyses ($P > 0.10$), which enabled an across line analysis to be conducted. A trend was detected ($P < 0.10$) for the haplotype effects on firmness with a difference of 0.08 ($P < 0.05$) between Haplotypes 1 and 3 (Table 3), and Haplotype 1 associated with a lower firmness. Haplotype 1 was also associated with less firmness compared with Haplotype 3 in the B × Y population. In addition, the differences between haplotype effects were relatively small for firmness. In this data set, we also tested the *Arg249Lys* and *Ser638Arg* substitutions separately. The overall significance for both polymorphisms was very close to the 0.05 significance level and LS means of genotypes across lines (Table 4) showed significant differences for firmness. The *249Lys/249Lys* and *638Arg/638Arg* genotypes were associated with lower firmness, which is in agreement with the results of the B × Y haplotype association analysis. The LS mean of the *249Lys/249Lys* genotype was different from the LS mean of *249Lys/249Arg* genotype ($P < 0.05$) and the *638Arg/638Arg* and *638Arg/638Ser* genotypes were different from the homozygote for the *638Ser* allele ($P < 0.05$).

Commercial Crossbred Pig Product Analysis. Haplotype association analysis was performed and the results

Table 2. Haplotype substitution effects for meat quality traits in B × Y F$_2$ animals

Trait[b]	Mean	SD	P[c]	Haplotype contrasts[a]		
				1 vs. 2	1 vs. 3	2 vs. 3
Firmness score	3.43	0.63	0.01	−0.23	−0.06	0.17
SE	0.03			0.08	0.04	0.08
P				0.006	0.1	0.04
Avg Instron force, kg	4.36	0.87	0.009	0.07	−0.14	−0.21
SE	0.04			0.11	0.05	0.11
P				0.53	0.006	0.06
Sensory tenderness score	7.85	1.15	0.17	0.04	0.14	0.1
SE	0.05			0.16	0.07	0.16
P				0.81	0.06	0.54
Sensory chewiness score	2.41	0.92	0.09	−0.1	−0.12	−0.02
SE	0.04			0.13	0.06	0.13
P				0.42	0.03	0.87
Sensory juiciness score	6.03	1.47	0.04	0.16	0.22	0.06
SE	0.07			0.19	0.09	0.19
P				0.41	0.01	0.74

[a]Haplotype 1: *249Lys-638Arg* (frequency = 0.50); Haplotype 2: *249Arg-638Arg* (0.07); Haplotype 3: *249Arg-638Ser* (0.43).

[b]Number of observations varied from 448 to 482.

[c]*P*-value for overall test of the effect of calpastatin haplotypes.

were again presented as haplotype substitution effects (Table 5). Haplotype effects were found for percentage of cooking loss ($P < 0.001$) and juiciness score ($P < 0.05$), trends were found for longissimus dorsi pH ($P < 0.10$) and shear force ($P < 0.10$) and effects for tenderness score were found consistent with the results from the B × Y population (Table 2).

The effect of Haplotype 1 was different from the effect of Haplotype 2 for shear force ($P < 0.05$) and percentage of cooking loss ($P < 0.001$; Table 5). Also the effect of Haplotype 1 differed from that of Haplotype 3 for longissimus dorsi pH ($P < 0.05$), percentage of cooking loss ($P < 0.001$), and subjective juiciness ($P < 0.01$). Haplotypes 1 and 3 tended to differ for subjective tenderness ($P < 0.10$). For all traits, Haplotype 1 was the favorable haplotype and it was associated with higher pH, better subjective tenderness and juiciness, and significantly lower shear force and percentage of cooking loss.

Phosphorylation Site Prediction

Using NetPhos 2.0, six potential adenosine cyclic 3′, 5′-phosphate-PKA phosphorylation sites were predicted in CAST, of which four were at serine AA residues. Experimentation has revealed that for most protein kinases, phosphorylation of serine is generally preferred over threonine (Kennelly and Krebs, 1991). Interestingly, *CAST Ser66Asn*, which was in complete linkage disequilibrium with *Arg249Lys*, and *Ser638Arg* both affect the PKA consensus sequence for two of these predicted phosphorylation sites (Figure 3). Specifically, *638Ser* may eliminate a predicted PKA recognition sequence, replacing the required arginine with a serine from the −2/−3 position relative to the potentially phosphorylated serine. In the L domain, the *66Ser* allelic variant introduces a potential PKA recognition sequence.

Discussion

The calpain system, a Ca^{2+}-activated protease family, plays an important role in postmortem tenderization of skeletal muscle due to its involvement in the degradation of important myofibrillar and associated proteins (Koohmaraie, 1992). Even though their substrates exclude major key structural proteins, such as actin, myosin, and α-actinin, there is significant evidence linking the calpains and, indirectly, calpastatin, a calpain inhibitor, to tenderization in beef, pork, and lamb (Sensky et al., 2001). Also Ca^{2+} stimulates contractions in prerigor muscle and may stimulate more rapid glycolysis and pH decline.

The results reported in this study provide important evidence in favor of the presence of new alleles/haplotypes of the *CAST* gene associated with several important meat quality traits in pigs. This conclusion is based on the following facts: 1) the known role of calpastatin as a calpain inhibitor; 2) observation of suggestive QTL for firmness, average Instron force and related

Table 3. Haplotype substitution effects for firmness across four commercial purebred lines

Trait[b]	Mean	SD	P[c]	Haplotype contrasts[a]		
				1 vs. 2	1 vs. 3	2 vs. 3
Firmness score	3.05	1.03	0.07	−0.06	−0.08	−0.01
SE	0.04			0.04	0.04	0.05
P				0.13	0.04	0.79

[a]Haplotype 1: *249Lys-638Arg* (frequency = 0.64); Haplotype 2: *249Arg-638Arg* (0.16); Haplotype 3: *249Arg-638Ser* (0.20).

[b]Number of observations = 767.

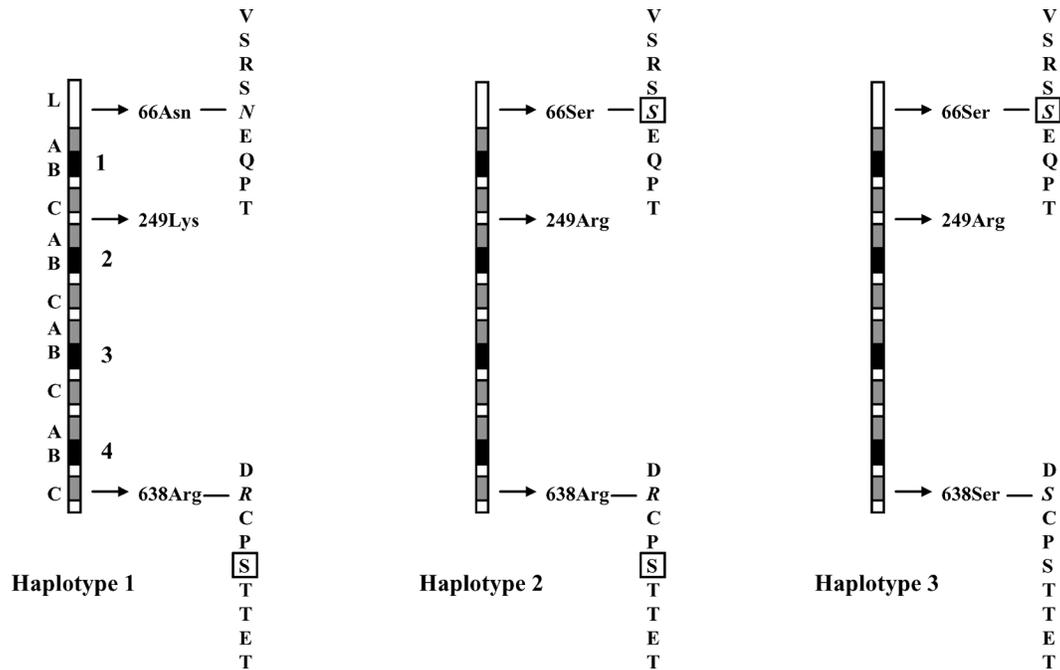[c]*P*-value for overall test of the effect of calpastatin haplotypes.

Figure 3. The identified calpastatin (*CAST*) haplotypes and their location in the area of adenosine cyclic 3′, 5′-monophosphate (cAMP)-dependent protein kinase recognition sequences (cAMP-dependent protein kinase consensus sequence $RX_{1-2}S/TX$). Squared serine represents the potential phosphorylated site. The identified major substitutions are represented in italics.

subjective traits such as juiciness, chewiness, and tenderness scores in a B × Y F_2 family on SSC2 in the region where the *CAST* gene is located; 3) association results between the identified substitutions and/or haplotypes that explain most of the *CAST* coding sequence variation, with a number of relevant meat quality traits in several different commercial pig lines and data sets; and 4) the potential effect of the *CAST Ser66Asn* and *Ser638Arg* substitutions on phosphorylation of CAST by PKA as a result of changes in the PKA recognition sequence.

The evidence of the suggestive QTL for Instron force and related subjective meat quality traits on the SSC2 in the B × Y resource family was followed by mapping of the *CAST* gene in the same linkage map. The position of *CAST* under the QTL peaks suggested that *CAST*

might be responsible for the observed phenotypic variation. By sequencing the entire coding region in B × Y F_3 individuals with extreme values for meat quality, eight mutations, including three missense mutations, were identified. It is interesting to note that the differences in allele frequencies at each polymorphic site between the Berkshire and Yorkshire founders of the intercross family were not extreme. This may explain why the QTL was only significant for firmness and the QTL effect was smaller than the haplotype substitution effect (Haplotype 1 vs. 2) from association analysis, as the model used evaluates the difference in effects associated with an average allele derived from each of the parental breeds.

Two of the missense mutations, *CAST Arg249Lys* and *Ser638Arg*, are located in or close to Subdomain C of

Table 4. Association results between calpastatin *Arg249Lys* and *Ser638Arg* genotypes and firmness score across four commercial purebred lines

Polymorphism	Least squares means by genotype			P^a
	249Lys/249Lys	*249Lys/249Arg*	*249Arg/249Arg*	
Arg249Lys	2.96 ± 0.06[y]	3.06 ± 0.05[z]	3.09 ± 0.07[yz]	0.06
No.[b]	319	359	102	
	638Arg/638Arg	*638Arg/638Ser*	*638Ser/638Ser*	
Ser638Arg	3.03 ± 0.05[y]	3.04 ± 0.05[y]	3.29 ± 0.11[z]	0.06
No.	495	249	30	

[a]Overall significance value for an effect of genotype.
[b]Number of observations.
[y,z]Estimates without a common superscript differ, $P < 0.05$.

Table 5. Haplotype substitution effects for several meat quality traits for commercial crossbred pigs

Trait[b]	Mean	SD	P^c	Haplotype contrasts[a]		
				1 vs. 2	1 vs. 3	2 vs. 3
Longissimus dorsi pH	5.73	0.18	0.08	0.03	0.05	0.03
SE	0.02			0.03	0.02	0.03
P				0.33	0.03	0.36
Shear force, kg	1.92	0.55	0.10	−0.17	−0.07	0.10
SE	0.04			0.08	0.07	0.08
P				0.03	0.27	0.21
Cooking loss, %	24.23	5.01	0.0002	−2.64	−1.92	0.73
SE	0.36			0.71	0.59	0.68
P				0.0003	0.001	0.29
Tenderness score	7.33	1.32	0.20	0.08	0.28	0.20
SE	0.10			0.19	0.16	0.18
P				0.69	0.09	0.28
Juiciness score	8.02	1.14	0.02	0.13	0.38	0.25
SE	0.08			0.16	0.14	0.16
P				0.44	0.007	0.11

[a]Haplotype 1: *249Lys-638Arg* (frequency = 0.48); Haplotype 2: *249Arg-638Arg* (0.21); Haplotype 3: *249Arg-638Ser* (0.31).
[b]Tenderness and juiciness scores are assigned using a subjective method; number of observations = 158 to 205.
[c]*P*-value for overall test of the effect of calpastatin haplotypes.

their respective domains (see Figure 2). This subdomain potentiates the inhibitory activity of CAST (Takano and Maki, 1999). It has been suggested that single mutations in conserved regions of any of the A or C subdomains might affect CAST activity (Ma et al., 1994), even though they do not have inhibitory properties like Subdomain B. Both these substitutions (*Arg249Lys* and *Ser638Arg*) are outside the most conserved area of Subdomain C: KPxxEDDxIDALSxDF (reviewed by Takano and Maki, 1999), but the *Ser638Arg* mutation is separated by just one AA from this sequence. Recently, Tompa et al. (2002) suggested that the A and C subdomains are activators of calpains, which, if confirmed, would open new directions for the study of the calpastatin-calpain system.

The *Ser66Asn* polymorphism is situated in Domain L. The function of this domain is not clear even though its sequence is well conserved between mammalian species. Recently, Hao et al. (2000) demonstrated the role of the L domain in regulation of L-type Ca^{2+} channels in guinea pig cardiac myocytes, suggesting a role of CAST in restoring Ca^{2+} channel activity, which facilitates Ca^{2+} influx and subsequently activation of calpain.

The major haplotypes observed can be separated into two groups based on sequence variation; one group is represented by Haplotypes 1 and 4 and is characterized by the presence of the same blocks of allelic variants for the adjacent substitutions located in the codons 194 to 211 and 237 to 249. The second group is represented by Haplotypes 2 and 3 (Figure 2). The association results presented do not include Haplotype 4, but significant contrasts are seen between Haplotype 1 and either Haplotype 2 or 3, and with one exception not within the group of Haplotypes 2 and 3. It will be interesting to investigate whether these two different haplotype

groups share a different genetic origin and whether the substitution effect of Haplotype 4 is similar to Haplotype 1. These haplotypes can be characterized by the missense variants identified in the L domain (*Ser66-Asn*) and the C subdomains in Domains 1 (*Arg249Lys*), 3 (*Ala504Thr*) and 4 (*Ser638Arg*). Haplotypes 2 and 3 differ at the substitution in Subdomain 4C with Haplotype 2 (in common with Haplotypes 1 and 4) having an arginine at this position, whereas Haplotype 3 has a serine. It is interesting to note that a serine is found at this position in human, bovine, rabbit, rat, and murine sequences. Haplotypes 2 and 3 also differ at position 306; however, this is regarded as conservative change (*Leu306Val*) that is unlikely to be significant. The other haplotypes differ from each other at two or three positions. For example, Haplotype 1 differs from the other three haplotypes in having *66Asn* in the L domain and from Haplotypes 2 and 3 by having *249Lys* at Subdomain 1C. Finally, Haplotypes 1 and 3 also differ at Subdomain 4C at the codon 638 substitution, where Haplotype 1 (and Haplotype 2) has an arginine instead of a serine in the case of Haplotype 3. These haplotypes can be summarized as NKAR, SRAR, SRAS, and SKTR at these positions (Haplotypes 1 to 4, respectively). Two of the polymorphisms were therefore sufficient to define the three major haplotypes present in the commercial breeds (Figure 2) and therefore the *CAST Arg249Lys* and *Ser638Arg* polymorphisms were used for both single substitution and haplotype association analyses.

In the B × Y family the haplotype analysis showed important differences between the effects of Haplotypes 1 and 3 for average Instron force, juiciness, and chewiness scores. For firmness, significant differences were revealed between the effects of Haplotypes 1 and 2 and also between Haplotypes 2 and 3. Haplotype 1 was dem-

onstrated to be the favorable haplotype, and it was associated with juicier and tender pork that was also less firm (Tables 2 and 3). Low firmness scores are often considered to be unfavorable because they are generally associated with extreme values such as those observed in PSE pork. In this case, lower firmness may relate more to the higher tenderness score; however, the LS means of the analyzed genotypes are in the acceptable range for firmness. Sensory tenderness, which did not show significant differences between the haplotypes did, however, show a similar hierarchy, with Haplotype 1 being most favorable. Haplotype 1 (NKAR) differs from Haplotype 3 (SRAS) at three of the substitutions, whereas it only differs from Haplotype 2 (SRAR) at the first two positions. Haplotypes 2 and 3 only differ at the last position (*Ser638Arg*). Thus, there is some agreement between the number of AA differences (for these three domains) and the size of the differences between the meat quality traits (Table 2). Finally, similar results were obtained with commercial crossbred pigs, with Haplotype 1 being the favorable haplotype for loin pH, shear force, percent cooking loss and subjective tenderness and juiciness, with the most significant difference been between Haplotypes 1 and 3 (except for shear force and percent cooking loss; Table 5).

In the single allele substitution association analyses, *P*-values very close to significance (0.05) were obtained for both substitution sites (Table 4). Significant differences were obtained between the LS means estimated for each *CAST* genotype, with the *249Lys/249Lys* and *638Arg/638Arg* genotypes being associated with a lower firmness as expected. In the 249 codon substitution analysis, Haplotype 1 was contrasted with Haplotypes 2 and 3, and in the 638 substitution analysis, Haplotype 3 was contrasted with Haplotype 1 and 2. Although initial examination of these observations (Table 4) points to the larger effects being associated with the substitution at codon 638, some caution is needed because genotype comparisons have considerable differences in numbers of observations.

The associations observed mean that selecting for Haplotype 1 could have a significant effect on meat quality. The effect of Haplotype 1 on percent cooking loss is estimated to correspond to a decrease of 2.64% per copy of Haplotype 1 compared with Haplotype 2. For shear force, an effect of 0.17 kg per copy of Haplotype 1 was detected (even after 14 d of aging) compared with Haplotype 2. Significant differences were also found between Haplotypes 1 and 3 for longissimus dorsi pH (0.05 per copy), percent cooking loss, and subjective juiciness (0.38 per copy). The directions of the effects of the different traits follow the known correlations for these traits, supporting the suggestion that the observed haplotype associations are real.

Using an additional small data set of 64 crossbred Duroc and Yorkshire animals a significant effect was seen of the *CAST Arg249Lys* and *Ser638Arg* on Warner-Bratzler Shear force at d 3 and 5 postmortem. At d 5,

an effect of 0.72 kg per copy of the 638Arg allele was observed (data not shown).

A comparison of *CAST* sequences from different mammals shows that the sequence around the putative phosphorylation sites is reasonably well conserved. For example, for the *Ser638Arg* position a serine is found in sequences of human (GenBank, BAA03747), cattle (AAA19643), rabbit (AAA31186), and mouse (BAA84768), although an alanine is present in sheep (AAB07483). At position 66, a serine or asparagine is present in pigs, and in this case, both AA have been reported with a serine in the case of sheep and cattle, asparagine in the mouse, and alanine in humans. However, the pig sequence is unique in that it is predicted to have full recognition sequences at these two sites for PKA.

Calpastatin solubility and cellular location are influenced by phosphorylation by PKA (Averna et al., 2001). Adachi et al. (1991) showed that phosphorylation increased the proportion of CAST bound to membranes and Salamino et al. (1997) demonstrated that phosphorylation decreased CAST inhibitory efficiency. Calpastatin phosphorylation could therefore influence proteolysis and ultimately have an effect on tenderness and other related meat quality traits. Analysis of the variation in porcine *CAST* suggests that both *CAST Ser66-Asn* and *Ser638Arg* could affect a PKA recognition consensus sequence. Haplotype 1 is the only haplotype (of Haplotypes 1 to 3) that has the *66Asn* variant (as well as *249Lys*), and it may therefore encode a peptide that will not be phosphorylated at the 66th AA (Figure 3). Haplotype 1 also contains an arginine at position 638, as part of the potential PKA recognition sequence, which might promote phosphorylation. Cong et al. (1998) demonstrated PKA phosphorylation of a truncated cattle CAST peptide consisting of Domains 1 to 4, which includes codon 638. Results from our association analysis demonstrate that Haplotype 1 is associated with higher tenderness and juiciness scores and this might be a result of advanced proteolysis of several key proteins. Haplotype 3 differs from Haplotype 1 at both of the potential phosphorylation sites discussed here (at AA 66 and 638), and encodes a peptide that will promote phosphorylation based on the *66Ser* allele but not based on 638Ser. For this reason, *Ser638Arg* might be an explanation for the significant differences observed between Haplotypes 1 and 3. Based on this hypothesis, we expected to see the same differences between Haplotypes 2 (which has same *638Arg* variant, like Haplotype 1) and 3, but did not. Therefore, we cannot attribute all of the differences to 638 substitution, and a different hypothesis must be proposed. Further biochemical study will be needed to test whether decreased PKA phosphorylation does occur at the CAST *66Asn* and/or CAST *638Ser* sites. The other substitution contained in the main haplotypes is at position *Arg249Lys*, and an arginine is found at this position in human, ovine, bovine, rabbit, rat, and murine sequences. It is not clear at this point which of the ob-

served variants may be causative as the haplotypes contain more than one different substitution variant, and the associations also may be due to undetected genetic variation in the regulatory regions of CAST or by genes whose effects have been captured by haplotype analysis due to linkage disequilibrium. Two of the variants identified, *Ser66Asn* and *Ser638Arg*, based on their position, AA change, and potential effect on PKA phosphorylation of CAST, may influence postmortem proteolysis. At least in the commercial lines, the *Ser66-Asn* polymorphism was in linkage disequilibrium with the *Arg249Lys* polymorphism, even though they are located a significant distance apart in genomic DNA. Of the haplotypes present in these lines, the variants *66Asn* and *249Lys* of these substitutions are specific to Haplotype 1. The *Ser66Asn* is a nonconservative substitution and is located in a conserved area of Domain L. This may suggest an important functional role that might be associated with the observed phenotypic differences in meat quality.

Implications

In recent years, several meat quality markers that have contributed significantly to pork quality, including Halothane and Rendement Napole, have been identified. This is the first study to describe genetic variation in the entire coding region of the porcine calpastatin gene, and results suggest that several substitutions may be associated with significant variation in meat quality traits. The newly identified calpastatin variants have significant effects on tenderness and other commercially important pork quality traits. It remains to be further demonstrated whether the effects are caused by these substitutions alone or by their linkage disequilibrium with the causative mutations. The identified genetic polymorphism can be used in breeding programs to improve overall meat quality and, thereby the economic value for the pork supply chain and quality products for consumers.

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