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Relationships among *calpastatin* single nucleotide polymorphisms, *calpastatin* expression and tenderness in pork longissimus[1]

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Summary

Genome scans in the pig have identified a region on chromosome 2 (SSC2) associated with tenderness. *Calpastatin* is a likely positional candidate gene in this region because of its inhibitory role in the calpain system that is involved in postmortem tenderization. Novel single nucleotide polymorphisms (SNP) in *calpastatin* were identified and used to genotype a population (n = 1042) of Duroc–Landrace–Yorkshire swine for association with longissimus lumborum slice shear force (SSF) measured at days 7 and 14 postmortem. Three genetic markers residing in the *calpastatin* gene were significantly associated with SSF ($P < 0.0005$). Haplotypes constructed from markers in the *calpastatin* gene were significantly associated with SSF (F-ratio = 3.93; P-value = 0.002). The levels of normalized mRNA expression of *calpastatin* in the longissimus lumborum of 162 animals also were evaluated by real-time RT-PCR and were associated with the genotype of the most significant marker for SSF ($P < 0.02$). This evidence suggests that the causative variation alters expression of calpastatin, thus affecting tenderness. In summary, these data provide evidence of several significant, publicly available SNP markers associated with SSF that may be useful to the swine industry for marker assisted selection of animals that have more tender meat.

Keywords calpastatin, gene expression, meat quality, pig, single nucleotide polymorphism.

Introduction

The quality and palatability of fresh retail pork are variable and pricing is not adjusted, regardless of quality (Wright *et al.* 2005). Efforts to improve the consistency and quality of fresh retail pork would improve consumer acceptance and perception of pork. Tenderness, juiciness, colour, flavour and cost are key factors guiding consumer choices for pork. The ability to select genetically for animals that are superior and consistent for meat quality traits would benefit the pork industry. Accurate markers to identify animals with superior meat quality may increase consumer appeal for pork and aid producer attempts to market branded products.

Pork tenderness is a highly heritable trait (h^2 = 0.45) in both Duroc and Landrace pigs (Lo *et al.* 1992; Suzuki *et al.* 2005). However, the genetic components for meat tenderness are complex and are controlled by multiple genes, as several quantitative trait loci (QTL) for tenderness measures have been identified (Malek *et al.* 2001; Rohrer *et al.* 2006; Edwards *et al.* 2008). One QTL region in particular has been detected on SSC2 in at least three different commercial-type pig populations (Stearns *et al.* 2005; Rohrer *et al.* 2006; Meyers *et al.* 2007). A Duroc–Landrace F_2 population revealed two QTL for longissimus lumborum slice shear force (SSF) on SSC2 with a suggestive QTL for SSF at 2 days postmortem positioned over the *calpastatin* (*CAST*) gene (Rohrer *et al.* 2006).

Calpastatin is the specific endogenous inhibitor of calpains, which are calcium-dependent proteases responsible for postmortem tenderization of meat (Koohmaraie 1992).

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A previous study found that a haplotype constructed with three single nucleotide polymorphisms (SNPs) in the coding region of *CAST* was associated with the meat quality traits of Warner–Bratzler shear force, cooking loss and juiciness (Ciobanu *et al.* 2004). Furthermore, a recent haplotype analysis in the University of Illinois meat quality pedigree population reduced the QTL interval for tenderness on chromosome 2–1.8 cM containing the *CAST* gene (Meyers *et al.* 2007). These studies provide additional evidence that *CAST* is a likely candidate affecting tenderness in this region.

The aims of this study were to investigate the region of chromosome 2 for its role affecting SSF and specifically, to evaluate the possible involvement of *CAST* and surrounding genes on chromosome 2 in pork tenderness. The markers presented here are predictive of longissimus lumborum tenderness in a Duroc–Landrace–Yorkshire population and could provide producers with beneficial markers for pork tenderness in other commercial swine populations.

Materials and methods

Animal care

Animals were fed a corn–soybean meal grower ration of 16% protein up to 16 weeks of age and 15% protein after 16 weeks of age (Klindt *et al.* 2006). Diets were formulated to meet or exceed National Research Council recommendations (NRC, 1998). All animal procedures were reviewed and approved by the US Meat Animal Research Center Animal Care and Use Committee and procedures for handling pigs complied with those specified in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

Resource population and phenotyping

The phenotyped animals were from a multigenerational population developed at the US Meat Animal Research Center (USMARC) by mating Yorkshire–Landrace composite females to either Duroc or Landrace boars selected from the industry. Twelve boars of each breed were randomly assigned a sire code and mated to Yorkshire–Landrace females (n = 220). This population (2001-born animals) was further developed by mating female descendants (n = 10/boar) to either a Duroc or Landrace-sired boar; Duroc-sired animals were mated to Landrace-sired animals. Subsequent matings were random except that matings of the same sire line code were avoided. Approximately 10 barrows per boar from the Duroc or Landrace-sired matings with Yorkshire–Landrace composite females (2001-born animals; n = 24 boars, 12 boars per breed), 60 barrows and gilts per boar (2004-born animals; n = 3 boars), 40 gilts per boar (2005-born animals; n = 12 boars) and 10 barrows per boar (2006-born animals; n = 12 boars) were harvested and phenotyped (Table 1). All animals were harvested at the USMARC abattoir and phenotypes were collected as described in Rohrer *et al.* (2006).

Table 1 Description of the USMARC resource population and phenotypic mean values.

Year[1]	Animals[2]	SSF (kg)[3]	Range (kg)
2001	227	11.28 ± 2.77	6.8–28.1
2004	163	14.32 ± 2.92	9.1–24.6
2005	532	13.87 ± 3.53	6.4–29.4
2006	120	14.66 ± 4.44	8.6–31.9
Total	1042	13.47 ± 3.61	6.4–31.9

[1]The year animals were born.

[2]Animals with phenotypes available.

[3]Slice shear force (SSF; mean ± SD) for years 2001, 2005 and 2006 was measured at 14 days postmortem. Slice shear force in year 2004 was measured at 7 days postmortem.

The longissimus was removed from pork carcasses and frozen at −20 °C. Tenderness as SSF was assessed at either day 7 or 14 postmortem according to Shackelford *et al.* (2004). Briefly, chops were thawed until an internal temperature of 5 °C was reached. Belt grill cooking was conducted with a Magigrill (model TBG-60; MagiKitch'n Inc.). Belt grill settings (top heat = 163 °C, bottom heat = 163 °C, preheat = 149 °C, gap between platens = 2.16 cm, and cook time = 5.8 min) were designed to achieve a final internal temperature of 71 °C for 2.54-cm-thick longissimus chops. Immediately after cooking, a 1-cm-thick, 5-cm-long slice was removed from each chop parallel to the muscle fibres. The slice was acquired by first cutting across the width of the longissimus at a point approximately 2 cm from the lateral end of the muscle. Using a sample sizer, a cut was made across the longissimus parallel to the first cut at a distance 5 cm from the first cut. Using a knife that consisted of two parallel blades spaced 1 cm apart, two parallel cuts were simultaneously made through the length of the 5-cm-long chop portion at a 45° angle to the long axis of the longissimus and parallel with the muscle fibres. Each sample was sheared once with a flat, blunt-end blade using an electronic testing machine (model 4411; Instron Corp.). The crosshead speed was set at 500 mm/min. Table 1 is a summary of the SSF values collected including the mean values, ranges and standard deviations for each group of animals in the population used in this study.

Identification of bacterial artificial chromosome clones

Bacterial artificial chromosomes (BACs) containing the *CAST* gene were identified from the CHORI-242 library (BACPAC Resources) by probing filters using random-primer labelled PCR products representing exons 1 (1u) and 30. CHORI-242 clones 401F7 and 111C3 were subcloned by digestion with BamHI and HindIII and ligation into pBluescript. One 96-well plate of clones from each library was sequenced with T3 and T7 primers. Contigs were built

using Consed and contigs were aligned to the pig *CAST* mRNA (GenBank M20160). Another library was sample sequenced by pooling the two BAC clones above and partially digesting with Sau3AI. A BAC containing *adenosine monophosphate deaminase 3* (*AMPD3*), CHORI-242 clone 53P13, was identified from the pig BAC Map (http://www.sanger.ac.uk/Projects/S_scrofa/mapping.shtml) and sample sequenced as above.

Identification of SNP markers

Primer pairs for amplification of *CAST* from genomic DNA were designed from porcine sequences obtained from BAC subclone sequences and those deposited in GenBank (Accession numbers CU467752 and CU469214 for *CAST*) using PRIMER 3 (Rozen & Skaletsky 2000) (code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). Primers for other loci were designed from available EST, genomic or BAC-end sequences. Primer sets were obtained from IDT (Integrated DNA Technologies). PCR was performed in a PTC-225 DNA engine (MJ Research Inc.) using 0.5 U Hot Star Taq polymerase (Qiagen); 1× supplied buffer; 1.5 mM $MgCl_2$; 200 μM dNTPs; 0.8 μM each primer; and 100 ng genomic DNA of eight animals representing different sire lines in 25 μl reactions. Five microlitres of the PCR reaction was electrophoresed in 1.5% agarose gels to determine quality of amplification and the remainder was prepared for sequencing after treatment with 0.1 U exonuclease I (USB). Sequencing reactions were precipitated with 58% isopropanol and sequenced with an ABI 3730 capillary sequencer (Applied Biosystems). Bases were called with Phred and assembled into contigs with Phrap. Polymorphisms were identified using POLYPHRED and assessed using Consed (http://www.phrap.org).

Genotyping analysis

Multiplex assays for use in the Sequenom MASSARRAY® system were designed using MASSARRAY ® Assay Design software. Assays were designed for 40 SNPs on chromosome 2. Each amplification primer had a 10-base tag added to ensure that the amplification primer masses were outside the range of the allele masses. Amplicon lengths were approximately 120 bp. Reaction conditions were performed as suggested by Sequenom iPLEX chemistry.

Association analyses

The data were analysed using an animal model which included fixed effects of sex and harvest date, covariates of SNP regressors and random additive polygenic effects. Regressors (independent variables; number of allele substitutions) for additive, dominant and parent-of-origin effects of each SNP were calculated using genotypic probabilities generated by allelic peeling algorithms in GENOPROB (Thallman 2002) as described by Kuehn *et al.* (2007). Variance components for polygenic effects and error were estimated using MTDFREML (Boldman *et al.* 1995). Each SNP was fitted separately due to potential multi-colinearity between regressions of closely linked markers. Nominal significance values were computed. For the three most significant SNPs (41646_874i, 41650_892g and 41658_290i), a second analysis was conducted without the parent-of-origin effect, as it was not a significant source of variation for any of these markers and this genomic region is not known to contain imprinted genes. A correction for multiple testing was applied to markers with significant associations by multiplying the estimated *P*-value by the number of unique tests.

Haplotypes were developed for each animal in the pedigree for the three most significant SNPs (41646_874i, 41650_892g and 41658_290i), based on most likely ordered genotypes determined by GENOPROB (Thallman 2002). For <5% of the data, GENOPROB predicted rare haplotypic genotypes in progeny that were inconsistent with Mendelian segregation. It was assumed that the predicted ordered genotypes (determining which set of alleles were of maternal and paternal origin) were incorrect because a change in order of a single marker yielded a common haplotypic genotype for the progeny as well as fitting Mendelian segregation. None of the changes made altered the actual genotype of a marker; they only changed the estimated parental origin of an allele. A final association analysis was conducted fitting the haplotypic genotypes as a fixed effect in the animal model described above.

Linkage disequilibrium analysis and Hardy–Weinberg equilibrium

Linkage disequilibrium (LD) (r^2) was estimated for the 40 SNPs on chromosome 2 using HAPLOVIEW 4.0 software (Barrett *et al.* 2005; http://www.broad.mit.edu/mpg/haploview/index.php). Haplotype blocks were based on pairwise LD values. HAPLOVIEW was also used to test whether the allele frequencies of each SNP on SSC2 conformed to Hardy–Weinberg equilibrium (HWE) expectations and to identify tag SNPs. The program uses the PHWE test statistic to evaluate the integrity of the genotypic data (Wigginton *et al.* 2005).

Calpastatin gene expression

Total RNA was extracted from 50 mg porcine longissimus lumborum (n = 162) from animals born in 2006 and 2008 by homogenization with TriZol reagent (Invitrogen) following the manufacturer's protocol. RNA pellets were resuspended in 100 μl DEPC-treated water and absorbance was measured by spectrophotometry at 260 nm. RNA (2 μg) was reverse transcribed using oligo dT_{18} primer and M-MLV reverse transcriptase (Promega). Primers for *CAST*

(GenBank M20160) and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*, GenBank AF017079) were designed for real-time RT-PCR using PRIMER 3. Primers used to amplify *CAST* were located in exons 4 and 6. The *CAST* forward primer sequence (nucleotides 213–233) was 5′-AGGCTGTAAAAACAGAACCTG-3′ and the reverse primer sequence was 5′-ATTTCTCTGATGTTGGCTGCTC-3′ (nucleotides 392–413). The *GAPDH* forward primer sequence (nucleotides 739–757) was 5′-GCGTGAACCATGAGAAGTATGA-3′ and the reverse primer sequence (nucleotides 947–967) was 5′-GGTAGAAGCAGGGATGATGTTC-3′. Real-time PCR was performed using 1× iQ™ SYBR® Green Supermix (Bio-Rad), 0.4 ng of cDNA and 0.3 μM of each primer. The PCR reaction was performed at 95 °C for 3 min followed by 40 cycles at 95 °C for 20 s, 58 °C for 30 s and 70 °C for 1 s on a MJ PTC-200 with a Chromo-4 detector (MJ Research). To evaluate the amplification efficiencies of *CAST* and *GAPDH* fragments, real-time RT-PCR analysis was performed with serial dilutions of a pooled sample of cDNA from eight 2006 barrows. A linear relationship was established for the difference in cycle threshold (ΔC_t) values of target and reference genes from concentrations of cDNA ranging from 0.08 to 10 ng and a standard concentration of 0.4 ng of cDNA was chosen for the real-time assays. The threshold cycle (C_t) for *CAST* and *GAPDH* of each sample was determined and used to calculate the $\Delta\Delta C_t$ using a reference cDNA pool. The fold difference between samples was obtained using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001).

Analysis of gene expression data

Gene expression data were analysed using the QTL Association option of MENDEL 8.0.1 (Lange *et al.* 2001). The statistical model included the random effects of harvest date and real-time assay group as fixed effects.

Results

Slice shear force values were collected from four different generations of pigs produced at USMARC. SSF in longissimus lumborum was highly heritable in the USMARC swine population with a heritability estimate of 0.54, which is comparable to previously reported heritabilities for tenderness in other porcine populations (Lo *et al.* 1992; Suzuki *et al.* 2005). The numbers of animals phenotyped by year, the mean and range of SSF values, as well as the standard deviations, are presented in Table 1. These values are similar to those reported for SSF at 7 days postmortem (Rohrer *et al.* 2006).

Sequencing and polymorphism detection

Quantitative trait loci on SSC2 were identified for SSF in the region of the *CAST* gene (Rohrer *et al.* 2006). To investigate the role of this region in tenderness further, polymorphisms were detected in eight animals of different sire lines on chromosome 2 from 64.8 to 138 cM. This region covers the *CAST* gene and flanking genomic DNA regions. The entire *CAST* sequence was contained in the subclone sequences of two BAC clones (GenBank CU467752 and CU469214) from the Swine Genome sequencing effort. The coding region of each exon and the flanking introns were sequenced and a total of 80 SNPs and indels were identified in *CAST* (Table S1). Most of the polymorphisms detected were located in intronic regions (Table S1); however, fifteen SNPs were detected in exons of *CAST* (Table S1). Three of these SNP resulted in non-synonymous amino acid changes at p.Ser88Asn, p.Lys271Arg, and p.Ser660Arg (positions based on GenBank accession number M20160). These three SNPs had been previously reported as p.Ser66Asn, p.Lys249Arg and p.Ser638Arg (Ciobanu *et al.* 2004). Additional polymorphisms were found in the other positional candidate genes and in the genomic regions flanking *CAST* (Table 2).

SNP genotyping and association

Forty SNPs on SSC2 were used to genotype the USMARC Duroc–Landrace–Yorkshire population of swine with meat quality phenotypic data using the Sequenom MASSARRAY system. The markers, their corresponding positions and observed allele frequencies are shown in Table 2. In addition to six novel SNPs in *CAST*, the three SNPs (41646_874i, 41650_892g, and M20160.638aai) previously reported by Ciobanu *et al.* (2004) to alter the amino acid sequence of calpastatin at p.Ser88Asn, p.Lys271Arg and p.Ser660Arg (positions based on GenBank accession number M20160), were also incorporated into the assays. Greater than 95% of the samples analysed generated reliable genotypes for all markers assayed.

The proportion of the variation in SSF that can be attributed to the markers analysed on SSC2 in this study was 11.641% (data not shown). Of the nine markers that were genotyped in the *CAST* gene, five were highly significant for association with SSF (Table 3). However, after adjustment for multiple testing, only three of the markers in *CAST* were significant for SSF (Table 3). The most significantly associated marker that had the largest effect on SSF was 41658_290i. Markers in *CAST* displayed additive effects ranging from 0.520 to 0.759 kg shear force.

Two other markers analysed in regions flanking *CAST* were also associated with SSF (Table 3). One marker located at 83 cM (65565_349) is 2-Mb upstream from *CAST* on the BAC map and another marker located at 86 cM (65644_369), 13-Mb downstream of *CAST*, were significantly associated with SSF (Table 3). These markers displayed additive effects. Other markers further upstream at 68.4 and 69.9 cM (12801.1i and 21726_2 respectively) were also associated with SSF, but displayed dominant or

Table 2 Associations and comparative and physical map of markers genotyped on pig chromosome 2.

Marker[1]	*F*-ratio (add)[2]	*P*-value	*F*-ratio (AD)[3]	*P*-value	SSC	Pig position[4]	HSA	Human position[5]	BAC map position[6]	Gene or BES	Allele frequency	Accession number[7]	dbSNP ss#
44785.574i	0.01	0.92	0.19	0.83	2	64.8	11	10.43	48,741,815	*AMPD3*	C/T (0.02, 0.98)	BV677845	48398076
44785.814i	0.73	0.39	1.84	0.16	2	64.8	11	10.43	48,741,815	*AMPD3*	C/T (0.45, 0.55)	BV677845	48398074
44791.142i	0.20	0.65	0.25	0.78	2	64.8	11	10.43	48,741,815	*AMPD3*	A/G (0.23, 0.77)	BV677848	48398085
45777.840i	0.92	0.34	1.79	0.17	2	64.8	11	10.43	48,741,815	*AMPD3*	A/G (0.90, 0.10)	BV677849	48398095
21726_2	0.10	0.75	2.96	0.052	2	68.4	19	3.60	56,866,000	*PIP5K1C*	C/T (0.52, 0.48)	BV677887	23132004
12801.1i	0.14	0.71	3.92	0.02	2	69.9	19	4.40	57,200,000	*CHAF1A*	A/G (0.90, 0.10)	BV103561	23129637
15971.1i	0.07	0.79	2.20	0.11	2	70.0	19	8.00	59,600,000	*FBN3*	C/T (0.18, 0.82)	BV103360	23129941
SW776					2	70.0	19						
13641.2i	1.28	0.26	1.54	0.21	2	70.0	19	13.10	63,688,000	*STX10*	A/G (0.11, 0.89)	BV103646	23130499
13943.1i	0.42	0.52	0.59	0.55	2	70.0	19	15.10	67,700,000	*SYDE1*	A/G (0.09, 0.91)	BV104551	23130298
65587_237	0.02	0.89	0.24	0.79	2	70.0	19	18.76	70,424,744	*COMP*	C/T (0.45, 0.55)	BV728496	95215175
65587_379	0.68	0.41	0.45	0.64	2	70.0	19	18.76	70,424,744	*COMP*	A/G (0.27, 0.73)	BV728496	95215177
27516.1i	0.27	0.6	0.23	0.79	2	70.0	1	226.20	74,350,000	*WNT9A*	A/G (0.43, 0.57)	BV102889	23132887
7241.1i	0.58	0.45	0.37	0.69	2	73.3	5	72.80	79,000,000	*BTF3*	A/T (0.77, 0.23)	BV729072	104806882
SW766					2	78.4	5	90.00	95,496,056				
65565_349	5.72	0.017	4.08	0.017	2	83.0	5	94.05	100,378,079	*MCTP1*	C/T (0.40, 0.60)	BV728494	95215173
41642_192i	8.26	0.0041	4.42	0.012	2	84.8	5	96.09	102,270,790	*CAST*	C/T (0.82, 0.18)	BV677806	48397977
41642_408i	9.27	0.0024	4.86	0.008	2	84.8	5	96.09	102,270,790	*CAST*	A/G (0.82, 0.18)	BV677806	48397975
41646_595g	6.74	0.0096	3.50	0.031	2	84.8	5	96.09	102,270,790	*CAST*	C/T (0.70, 0.30)	BV677807	48397984
41646_874i	14.88	0.00012	7.94	0.0004	2	84.8	5	96.09	102,270,790	*CAST*	A/G (0.43, 0.57)	M20160	
41650_892g	13.66	0.00023	7.25	0.0008	2	84.8	5	96.11	102,270,790	*CAST*	A/G (0.52, 0.48)	BV677894	48398421
41650_975g	6.01	0.014	3.19	0.042	2	84.8	5	96.11	102,270,790	*CAST*	C/T (0.80, 0.20)	BV677894	48398420
41658_290i	16.19	0.00006	8.10	0.0003	2	84.8	5	96.12	102,270,790	*CAST*	C/T (0.76, 0.24)	BV677813	48397998
23795.1i	1.95	0.16	3.07	0.047	2	84.8	5	96.12	102,270,790	*CAST*	C/T (0.65, 0.35)	BV103090	23131799
M20160.638aai	0.06	0.81	0.70	0.5	2	84.8	5	96.13	102,270,790	*CAST*	A/C (0.78, 0.22)	M20160	
SW1320					2	84.8	5						
65555_45	0.22	0.64	0.88	0.42	2	85.0	5	97.03	103,492,284	bE133I6	G/T (0.72, 0.28)	BV728493	95215172
S0010					2	85.9	5						
65644_139	1.08	0.3	1.61	0.2	2	86.0	5	108.70	114,902,973	*PJA2*	G/T (0.65, 0.35)	BV728502	95215192
65644_369	2.36	0.13	2.53	0.08	2	86.0	5	108.70	114,902,973	*PJA2*	A/G (0.36, 0.64)	BV728502	95215193
65636_270	0.63	0.43	0.34	0.71	2	87.0	5	111.66	118,000,000	*EPB41L4A*	C/T (0.70, 0.30)	BV728500	95215187
65626_380	3.23	0.073	2.73	0.66	2	88.0	5	113.93	119,678,009	*KCNN2*	A/C (0.49, 0.51)	BV728499	95215185
65626_395	2.84	0.092	1.84	0.16	2	88.0	5	113.93	119,678,009	*KCNN2*	A/G (0.51, 0.49)	BV728499	95215186
SW1695					2	88.5	5	115.00	121,389,310				
65650_316	0.09	0.76			2	89.0	5	116.20	122,030,292	bT214J3	A/G (0.46, 0.54)	BV728503	95215194
64271_45	2.54	0.11	1.27	0.28	2	93.0	5	121.43	126,869,986	*LOX*	C/T (0.11, 0.89)	BV728479	95215133
64269_254	0.58	0.45	1.64	0.19	2	93.0	5	121.43	126,869,986	*LOX*	A/C (0.16, 0.84)	BV728478	95215130
64269_459	1.91	0.17	0.95	0.39	2	93.0	5	121.43	126,869,986	*LOX*	C/T (0.18, 0.82)	BV728478	95215132
64267_56	1.24	0.27	0.62	0.54	2	93.0	5	121.44	126,869,986	*LOX*	C/T (0.18, 0.82)	BV728477	95215128
64259_163	1.43	0.23			2	93.0	5	121.44	126,869,986	*LOX*	C/T (0.82; 0.18)	BV729065	104806883

Table 2 Continued.

Marker[1]	*F*-ratio (add)[2]	*P*-value	*F*-ratio (AD)[3]	*P*-value	SSC	Pig position[4]	HSA	Human position[5]	BAC map position[6]	Gene or BES	Allele frequency	Accession number[7]	dbSNP ss#
SW1408					2	96.3	5						
65662_329	0.08	0.78	1.10	0.33	2	98.0	5	127.56	133,156,772	bT204L21	G/T (0.81, 0.19)	BV728506	95215198
SW1879					2	108.8	5						
16961_1	1.99	0.16	1.51	0.22	2	138.0	5	149.29	154,750,000	*PDE6A*	C/T (0.49, 0.51)	BV677882	48398332
65589_163	0.61	0.43	0.53	0.59	2	138.0	5	149.31	154,774,797	bE10O24	A/G (0.50, 0.50)	BV728497	95215178
65589_224	0.60	0.44	0.63	0.53	2	138.0	5	149.31	154,774,797	bE10O24	G/T (0.51, 0.49)	BV728497	95215179
65591_338	2.13	0.15	1.37	0.25	2	138.0	5	149.43	154,696,186	bE78G4	C/T (0.47, 0.53)	BV728498	95215183

[1]Marker numbers are derived from the corresponding primer numbers in the USMARC database.
[2]*F*-ratio calculated with regressors for additive effects.
[3]*F*-ratio calculated with regressors for additive and dominant effects.
[4]Most likely marker order based on the USMARC linkage map and the RH Map (Meyers *et al.* 2005).
[5]Human positions are in megabases and were obtained from the Human (*Homo sapiens*) Genome Browser Gateway based on NCBI Build 35 (http://genome.ucsc.edu/cgi-bin/hgGateway).
[6]BAC map positions are in basepair and were obtained from BAC end sequences that aligned to the sequences generated from the amplicons containing the markers. These positions were based on the Wellcome Trust Sanger Institute genome physical map (http://www.sanger.ac.uk/Projects/S_scrofa/mapping.shtml).
[7]Primer sequences are in accession numbers in GenBank.

codominant effects rather than the additive effect detected for markers located near or within the *CAST* gene (Fig. 1). After correction for multiple testing, none of these markers was significantly associated with SSF (Table 3).

Haplotype analysis

The SNPs genotyped in the *CAST* gene segregated into two haplotype blocks generated by HAPLOVIEW 4.0 (Fig. 2; haplotype blocks 2 and 3). Two of the markers, 41642_192i and 41642_408i, were identified in the same amplicon and were nearly in complete LD with each other (r^2 = 0.99). Markers 41646_874i and 41650_892g that alter the amino acid sequence of *CAST* were also detected in high LD (0.95). The marker with the highest association with SSF, 41658_290, was found in lower LD with the other *CAST* markers (0.35–0.73). Three tag SNPs; 41642_408i, 41646_874i and 41658_290i were predicted by HAPLOVIEW.

Three major haplotypes and one in low frequency were predicted by HAPLOVIEW 4.0 (in block 2) for *CAST* (Fig. 2). The most common haplotype, CACAACC, was detected at a frequency of 0.563. The frequency of the TGTGGTT haplotype, which was associated with higher SSF, was 0.171 (Fig. 2). *CAST* haplotypes constructed from markers 41646_874i, 41650_892g and 41658_290i, were evaluated by MTDFREML and were significantly associated with SSF (*F*-ratio = 3.93; *P*-value = 0.002). The haplotype analysis was less significant than the single marker association and did not explain additional phenotypic variation.

Relationship between *CAST* gene expression and SNPs in the *CAST* gene

To determine whether the expression of *CAST* was associated with *CAST* SNP genotypes, mRNA from longissimus lumborum of 2006 and 2008-born animals (n = 162) was evaluated for *CAST* mRNA levels by real-time RT-PCR. The quantity of *CAST* mRNA, calculated using the fold difference equation $2^{-\Delta\Delta C_t}$, was associated with 41658_290i (P = 0.011), the most significant SNP associated with SSF, and 41642_408i (Table 4). Twelve of these animals were homozygous T for the 41658_290i marker, 75 were heterozygous (C/T) and 75 were homozygous for the C allele. Table 4 shows the allelic effects of six SNPs in *CAST* on the relative gene expression of *CAST*.

Discussion

Genetic selection for increased lean growth in swine has led to decreased consumer acceptance because of a decrease in meat and eating quality of pork, including tenderness (Schwab *et al.* 2006). Feed sources and additives fed to pigs can also negatively affect meat quality (Carr *et al.* 2005). To make progress in improving these traits, the genetic sources of variation need to be identified. Techniques to measure

Table 3 *Calpastatin* marker associations, estimated additive effects and genotypic distribution.

Marker name[1]	Position in CAST	AA[2] (SE)	*F*-value[3]	*P*-value	Correction for multiple tests[4]	A allele frequency	AA[5]	Aa	aa
65565_349	−2 Mb	−0.418 (0.175)	5.72	0.02	NS	C = 0.40	0.147	0.498	0.355
41642_192i	Intron 2	0.605 (0.210)	8.26	0.004	NS	T = 0.18	0.035	0.298	0.667
41642_408i	Intron 3	0.641 (0.210)	9.27	0.002	NS	G = 0.18	0.035	0.292	0.673
41646_595g	Intron 5	0.626 (0.235)	6.74	0.01	NS	T = 0.30	0.037	0.152	0.811
41646_874i	Exon 6	0.587 (0.226)	14.88	0.0001	0.0033	G = 0.44	0.192	0.487	0.321
41650_892g	Exon 13	0.627 (0.170)	13.66	0.0002	0.0066	G = 0.48	0.177	0.480	0.344
41650_975g	Intron 13	0.520 (0.212)	6.01	0.01	NS	T = 0.20	0.034	0.280	0.686
41658_290i	Intron 19	0.759 (0.189)	16.19	0.00006	0.00198	T = 0.24	0.050	0.357	0.593
23795.1i	Intron 19	−0.226 (0.161)	1.95	0.2	NS	C = 0.65	0.414	0.461	0.125
M20160.638aai	Exon 26	−0.050 (0.186)	0.06	0.8	NS	C = 0.22	0.036	0.329	0.635
65644_369	+13 Mb	0.276 (0.180)	2.36	0.1	NS	G = 0.65	0.426	0.459	0.114

[1]Marker names derived from corresponding primer numbers in the USMARC database.
[2]Effect of AA genotype on slice shear force (kg) with the heterozygote (Aa) = 0.
[3]*F*-ratio calculated with regressor for additive effects.
[4]The correction for multiple tests was performed by multiplying the *P*-value from the association analysis with the number of unique markers (n = 33) where $r^2 \leq 0.95$ with other markers.
[5]Genotypic distribution.

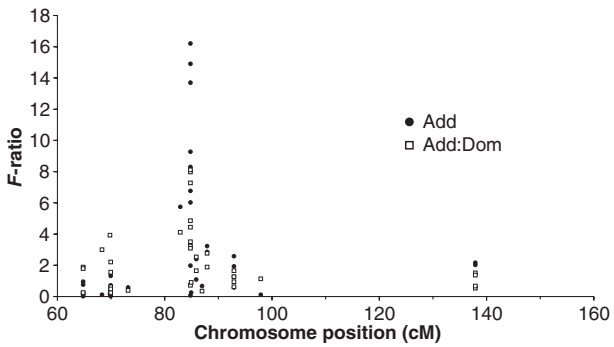

Figure 1 *F*-test statistics for slice shear force for each SNP marker on SSC2. The *x*-axis represents the relative position of each marker in cM and the *F*-ratio is shown on the *y*-axis. Each SNP was fitted separately due to potential multi-colinearity between regressions of closely linked markers. Open squares illustrate the *F*-test values calculated using regressors for additive and dominant effects. Black circles represent the *F*-test values calculated with an additive-only model.

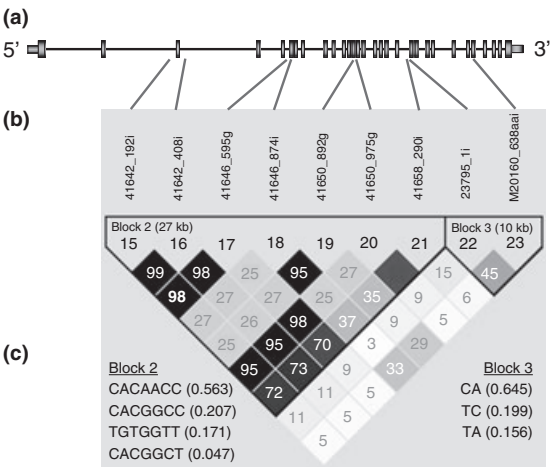

Figure 2 Linkage disequilibrium (LD) in the extended calpastatin region on chromosome 2. (a) Schematic of the *calpastatin* gene. Shaded rectangles illustrate exonic regions. The first exon depicted is exon 1u. Note: Exon 3 is illustrated, but is spliced out in the longissimus lumborum in pig. (b) HAPLOVIEW display of the LD for the SNP in the *calpastatin* gene. Pairwise LD (r^2) values are shown between markers. The shading represents the LD relationships with darker shading indicating higher LD. (c) HAPLOVIEW 4.0 haplotype display for markers genotyped in SSC2. HAPLOVIEW divided this region into seven blocks. Blocks 2 and 3 represent the SNPs in *calpastatin*; block 2 contains markers 15–21 and block 3 contains markers 22 and 23. The population frequencies are listed in parentheses next to each haplotype.

meat tenderness are invasive, disruptive to the processing of pork and labour-intensive (Shackelford *et al.* 2004). Identification of DNA markers that are predictive of these traits would substantially alleviate the problems and expense associated with measuring these traits. Sources of variation in pork tenderness include postmortem proteolysis, collagen concentration and sarcomere length (Wheeler *et al.* 2000). For these reasons, we chose to evaluate genes because of their location on chromosome 2 and their possible involvement in expression of these traits.

This study confirms the presence of a QTL for pork tenderness on chromosome 2 in another population of industry-relevant pigs. QTL for tenderness had previously been found on SSC2q and near the centromere of this chromosome (Stearns *et al.* 2005; Rohrer *et al.* 2006; Meyers *et al.* 2007). In an unrelated Duroc–Landrace population, Rohrer *et al.* (2006) identified QTL for SSF at day 7 and day 2 postmortem at 60 (near the centromere) and 79 cM respectively, with *CAST* mapping under the distal QTL. Meyers *et al.* (2007) showed evidence for two QTL for

Table 4 Associations between *calpastatin* gene expression and six SNPs in *calpastatin*.

Marker	Negative allele	Effect[1]	Positive allele	Effect	*P*-value
41642_192i	C	−0.1146	T	0.1146	0.0876
41642_408i	A	−0.1352	G	0.1352	0.0121
41646_874i	A	−0.0721	G	0.0721	0.2208
41650_892g	A	−0.0526	G	0.0526	0.3179
41650_975g	C	−0.1234	T	0.1234	0.0591
41658_290i	C	−0.1382	T	0.1382	0.0111

[1]Effect of genotype on the relative gene expression of *calpastatin*. Expression was calculated as fold difference ($2^{-\Delta\Delta C_t}$).

tenderness in a Berkshire–Duroc cross with one QTL being positioned at *CAST* and the other located more distal to this gene. Therefore, it seems likely that more than one QTL for tenderness resides on chromosome 2. The most significant associations were found within the *CAST* gene; however, association with markers 12801.1i, 21726_2, 65565_349 and 65644_369 support the possibility that other genes in this region on SSC2 may also be responsible for tenderness variation in pork longissimus lumborum. Markers in *AMPD3* and *lysyl oxidase* (*LOX*), at 64.8 and 93 cM respectively were evaluated because *AMPD3* and *LOX* were positional candidate genes. SNPs in *AMPD3* have been correlated to pH in broiler chicken breast muscle (El Rammouz *et al.* 2004). *LOX* facilitates collagen and elastin crosslinking and mutations in *LOX* have been associated with human connective tissue disorders. Our SNP analysis indicates that neither of these genes is associated with SSF in this population of animals.

Our efforts to sequence the entire *CAST* coding region to enable the identification of mutations have produced only three previously reported SNPs that result in non-synonymous amino acid changes (Ciobanu *et al.* 2004). The relatively conservative nature of the p.Ser88Asn and p.Lys271Arg polymorphisms and evaluation by the POLYPHEN program (http://genetics.bwh.harvard.edu) suggest that these are not likely to be causative mutations. The p.Ser660Arg polymorphism (marker M20160.638aai) was predicted as a possibly damaging amino acid alteration by POLYPHEN. However, marker M20160.638aai was not associated with shear force, indicating that p.Ser660Arg is unlikely to be a mutation affecting pork tenderness.

We identified markers in the *CAST* gene that are significantly correlated with SSF; the most significant marker was 41658_290i, a novel SNP found in intron 19. A recent report by Meyers & Beever (2008) presented nearly 900 SNPs in the *CAST* gene; however, the marker at 41658_290i was not detected in the IMQP population of animals, which suggests that 41658_290i is not the mutation for meat quality in pork. While none of the markers evaluated in this study is likely to be causative mutations for tenderness, the high association values suggest that they are in LD with the causative mutation.

To our knowledge, this is the first study demonstrating a genotypic effect on *CAST* mRNA expression. As the most significant marker for SSF, 41658_290i, was associated with the expression of *CAST* mRNA in this study and the fact that recent data by Meyers & Beever (2008) illustrated that the block of heterozygosity concordant with meat tenderness was in the 5′ region containing exons 1×a, 1×b, 1y, 1z and 1u, it seems likely that the causative mutation resides in a regulatory region. This implies that the causative mutation(s) in *CAST* associated with tenderness may affect the amount of *CAST* mRNA and calpastatin protein, either by regulating expression, by affecting mRNA stability or half-life, or by alternative splicing rather than the amino acid sequence of the protein. While identification of the causative variation will provide the most reliable marker for pork tenderness, characterization of the genetic architecture of this region could provide markers that are predictive of pork tenderness.

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References

Barrett J.C., Fry B., Maller J. & Daly M.J. (2005) HAPLOVIEW: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21,** 263–5.

Boldman K.G., Kriese L.A., Van Vleck L.D., Van Tassel C.P. & Kachman S.D. (1995) *A Manual for the Use of* MTDFREML. A Set of Programs to Obtain Estimates of Variance and Covariances (Draft). USDA, ARS, Lincoln, NE.

Carr S.N., Ivers D.J., Anderson D.B., Jones D.J., Mowrey D.H., England M.B., Killefer J., Rincker P.J. & McKeith F.K. (2005) The effects of ractopamine hydrochloride on lean carcass yields and pork quality characteristics. *Journal of Animal Science* **83,** 2886–93.

Ciobanu D.C., Bastiaansen J.W., Lonergan S.M., Thomsen H., Dekkers J.C., Plastow G.S. & Rothschild M.F. (2004) New alleles in *calpastatin* gene are associated with meat quality traits in pigs. *Journal of Animal Science* **82,** 2829–39.

Edwards D.B., Ernst C.W., Raney N.E., Doumit M.E., Hoge M.D. & Bates R.O. (2008) QTL mapping in an F_2 Duroc x Pietrain resource population: II. Carcass and meat quality traits. *Journal of Animal Science* **86,** 254–66.

El Rammouz R., Berri C., Le Bihan-Duval E., Babilé R. & Fernandez X. (2004) Breed differences in the biochemical determinism of ultimate pH in breast muscles of broiler chickens – a key role of AMP Deaminase? *Poultry Science* **83,** 1445–51.

FASS (1999) *Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching*, 1st edn. Federation of Animal Science Society, Savoy, IL.

Klindt J., Thallman R.M. & Wise T. (2006) Effects of sire line, sire, and sex on plasma urea nitrogen, body weight, and backfat thickness in offspring of Duroc and Landrace boars. *Journal of Animal Science* **84,** 1323–30.

Koohmaraie M. (1992) The role of Ca(2+)-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. *Biochimie* **74,** 239–45.

Kuehn L.A., Rohrer G.A., Nonneman D.J., Thallman R.M. & Leymaster K.A. (2007) Detection of single nucleotide polymorphisms associated with ultrasonic backfat depth in a segregating Meishan × White Composite population. *Journal of Animal Science* **85,** 1111–9.

Lange K., Cantor R., Horvath S., Perola M., Sabatti C., Sinsheimer J. & Sobel E. (2001) MENDEL version 4.0: a complete package for the exact genetic analysis of discrete traits in pedigree and population data sets. *American Journal of Human Genetics* **69**(Suppl.), A1886.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods* **25 (4),** 402–8.

Lo L.L., McLaren D.G., McKeith F.K., Fernando R.L. & Novakofski J. (1992) Genetic analyses of growth, real-time ultrasound, carcass, and pork quality traits in Duroc and Landrace Pigs: II. Heritabilities and correlations. *Journal of Animal Science* **70,** 2387–96.

Malek M., Dekkers J.C., Lee H.K., Baas T.J., Prusa K., Huff-Lonergan E. & Rothschild M.F. (2001) A molecular genome scan analysis to identify chromosomal regions influencing economic traits in the pig. II. Meat and muscle composition. *Mammalian Genome* **12,** 637–45.

Meyers S.N. & Beever J.E. (2008) Investigating the genetic basis of port tenderness: genomic analysis of porcine *CAST*. *Animal Genetics* **39,** 531–43.

Meyers S.N., Rogatcheva M.B., Larkin D.M., Yerle M., Milan D., Hawken R.J., Schook L.B. & Beever J.E. (2005) Piggy-BACing the human genome II. A high-resolution, physically anchored, comparative map of the porcine autosomes. *Genomics* **86,** 739–52.

Meyers S.N., Rodriguez-Zas S.L. & Beever J.E. (2007) Fine-mapping of a QTL influencing pork tenderness on porcine chromosome 2. *BMC Genetics* **8,** 69.

NRC (1998) *Nutrient Requirements of Swine*, 10th rev. edn. National Academic Press, Washington, DC.

Rohrer G.A., Thallman R.M., Shackelford S., Wheeler T. & Koohmaraie M. (2006) A genome scan for loci affecting pork quality in a Duroc–Landrace F_2 population. *Animal Genetics* **37,** 17–27.

Rozen S. & Skaletsky H.J. (2000) PRIMER3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (Ed. by S. Krawetz & S. Misener), pp. 365–86. Humana Press, Totowa, NJ.

Schwab C.R., Baas T.J., Stalder K.J. & Mabry J.W. (2006) Effect of long-term selection for increased leanness on meat and eating quality traits in Duroc swine. *Journal of Animal Science* **84,** 1577–83.

Shackelford S.D., Wheeler T.L. & Koohmaraie M. (2004) Technical note: use of belt grill cookery and slice shear force for assessment of pork longissimus tenderness. *Journal of Animal Science* **82,** 238–41.

Stearns T.M., Beever J.E., Southey B.R., Ellis M., McKeith F.K. & Rodriguez-Zas S.L. (2005) Evaluation of approaches to detect quantitative trait loci for growth, carcass, and meat quality on swine chromosomes 2, 6, 13, and 18. II. Multivariate and principal component analyses. *Journal of Animal Science* **83,** 2471–81.

Suzuki K., Irie M., Kadowaki H., Shibata T., Kumagai M. & Nishida A. (2005) Genetic parameter estimates of meat quality traits in Duroc pigs selected for average daily gain, longissimus muscle area, backfat thickness, and intramuscular fat content. *Journal of Animal Science* **83,** 2058–65.

Thallman R.M. (2002) *User*'s Manual for GENOPROB Version 2.000. USDA-ARS, U.S. Meat Animal Research Center, Clay Center, NE.

Wheeler T.L., Shackelford S.D. & Koohmaraie M. (2000) Variation in proteolysis, sarcomere length, collagen content, and tenderness among major pork muscles. *Journal of Animal Science* **78,** 958–65.

Wigginton J.E., Cutler D.J. & Abecasis G.R. (2005) A note on exact tests of Hardy–Weinberg equilibrium. *American Journal of Human Genetics* **76,** 887–93.

Wright L.I., Scanga J.A., Belk K.E. *et al.* (2005) Benchmarking value in the pork supply chain: characterization of US pork in the retail marketplace. *Meat Science* **71,** 451–63.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 GenBank accession numbers, primers and SNP positions for porcine *calpastatin*.

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