Predictive markers in calpastatin for tenderness in commercial pig populations

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Predictive markers in calpastatin for tenderness in commercial pig populations\textsuperscript{1,2}


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ABSTRACT: The identification of predictive DNA markers for pork quality would allow US pork producers and breeders to select genetically superior animals more quickly and efficiently for the production of consistent, high-quality meat. Genome scans have identified QTL for tenderness on SSC 2, which have been fine-mapped to the calpastatin locus. The objectives of this study were to identify the sequence variation in calpastatin that likely affects tenderness in commercial-level pig populations and to develop definitive DNA markers that are predictive of pork tenderness for use in marker-assisted selection programs. We resequenced the calpastatin regulatory and transcribed regions in pigs with divergently extreme shear force values to identify possible mutations that could affect tenderness. A total of 194 SNP were identified in this sequence, and 31 SNP were found in predicted transcription factor binding sites. We tested 131 polymorphisms in our research population and a subset (40) of these in samples of industry pigs for their association with objective measures of tenderness. We identified 4 SNP that were consistently associated with pork tenderness in all the populations studied, representing 2,826 pigs from 4 distinct populations. Gel shift assays were designed for these SNP and 12 other polymorphic sites. Six sites demonstrated a gel shift when probes were incubated with nuclear extract from muscle, heart, or testis. Four of these sites, a specificity protein 1 (Sp1) site around nucleotides 12978 and 12979, a potential thyrotroph embryonic factor (Tef) site at nucleotide 25587, an unknown site at nucleotide 48699, and myocyte enhancer factor-2 (Mef-2)/TATA sites with SNP at positions 49223 and 49228 were allele specific in binding nuclear proteins. The allele frequencies for the tender alleles were similar (0.11 to 0.36) in the 4 different commercial populations. These 4 SNP were not in complete linkage disequilibrium with each other and may independently affect calpastatin expression, tenderness, or both. These markers should be predictive of pork tenderness in industry populations.

Key words: calpastatin, pork, single nucleotide polymorphism, tenderness, transcription factor site

\textsuperscript{1}Mention of trade names or commercial products is solely for the purpose of providing information and does not imply recommendation, endorsement, or exclusion of other suitable products by the USDA.

\textsuperscript{2}The authors thank Sue Hauver and Kris Zimmerman (USMARC) for technical assistance, Sherry Kluver (USMARC) for manuscript preparation, and the National Swine Registry (West Lafayette, IN) for providing samples for genotyping. This research was funded by National Pork Board (Des Moines, IA) project numbers 06-139, 07-005, and 08-105. Projects 06-139 and 07-005 were managed through The Ohio State University Research Foundation (Columbus), with subcontract awards with the Texas AgriLife Research (Texas A&M University, College Station), the Iowa State University Office of Research Assurances (Ames), and the USDA, ARS, Northern Plains Area Natural Resources Research Center (Fort Collins, CO). Principal investigators for projects 06-139 and 07-005 were Steven J. Moeller (The Ohio State University), Rhonda K. Miller (Texas A&M University), and Henry Zerby (The Ohio State University). Project 08-105 was managed through USDA-ARS, US Meat Animal Research Center (Clay Center, NE; principal investigator, Dan Nonneman).

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INTRODUCTION

Pork tenderness is a highly heritable trait (Suzuki et al., 2005) and several QTL have been identified. One QTL in particular, found in at least 3 different commercial-type pig populations, resides on SSC 2 positioned over the calpastatin gene (Malek et al., 2001; Stearns et al., 2005; Rohrer et al., 2006; Meyers et al., 2007; Edwards et al., 2008; Meyers and Beever, 2008). Calpastatin is the specific endogenous inhibitor of calpains, calcium-dependent proteases responsible for postmortem tenderization of meat (Koohmaraie, 1992). Linkage and haplotype association analyses (Ciobanu et al., 2004; Meyers et al., 2007) provide further evidence that calpastatin is a likely candidate gene affecting tenderness in this region. We have also analyzed SNP in this region in commercial-type populations and have found that markers in the calpastatin gene are more significantly associated with slice shear force and calpastatin expression than the flanking markers (Lindholm-Perry et al., 2009). However, the significance of markers in calpastatin differs by marker and population (Rohrer et al., 2007). Identifying the causative mutation(s) in calpastatin would provide definitive markers for tenderness in all US swine populations. The objectives of this research were to identify possible SNP in the calpastatin gene causing the genetic variation responsible for the variation in tenderness, to evaluate these SNP for associations with slice shear force in our phenotyped composite population, and to validate SNP effects in commercial-level pigs obtained from the industry.

MATERIALS AND METHODS

All animal procedures were reviewed and approved by the US Meat Animal Research Center (USMARC) 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, 2010).

USMARC Resource Population

The animals were a multigenerational Duroc-Yorkshire-Landrace composite population developed at the USMARC as described before (Lindholm-Perry et al., 2009). The phenotyped animals included 2001-born barrows, 2005-born gilts, 2006-born barrows, and 2008-born barrows and gilts (n = 1,042). All animals were slaughtered at the USMARC abattoir, and phenotypes were collected as described before (Shackelford et al., 2004; Lindholm-Perry et al., 2009).

Industry F2 Population

This population was composed of 50 F2 Duroc-Landrace litters produced from 4 F1 boars and 50 F1 sows (Rohrer et al., 2006). A total of 370 animals phenotyped for slice shear force on d 2 and 7 postmortem were included in this study.

The Ohio State University National Pork Board Industry Samples

A total of 903 whole, boneless loins were obtained from 3 different plants at about 24 h postmortem (Moeller et al., 2010). A subset of loins (n = 227) from 1 plant were enhanced to improve tenderness. Samples were selected to represent the range of Minolta L* (lightness) color, 24-h ultimate pH, and marbling score. At d 7 postmortem, chops were cooked at 4 different temperatures (62, 68, 74, 79°C), and Warner-Bratzler shear force was measured. Genomic DNA was extracted from muscle using a Wizard SV 96 Genomic DNA Purification Kit (Promega, Madison, WI).

Babcock Genetics Industry Samples

Gilts from 3 different lines with pedigree information from Babcock Genetics (Rochester, MN) were slaughtered in groups of 25 at a commercial plant and then evaluated for pork quality by K. Prusa (Iowa State University, Ames). Chops (n = 535) were aged for 10 d, and instrumental tenderness was measured using an Instron Star probe (Instron, Norwood, MA). From separate chops, cooking loss, sensory tenderness, juiciness, flavor and off-flavor, and percentages of moisture and fat were collected. A 10-d purge weight; chop purge; 3 pH measurements (1 each from the blade region, center region, and back-end region, before the sirloin); and Minolta L*, a* (redness), and b* (yellowness) color scores were collected. Blood from 594 animals was collected on FTA (Fast Technology for Analysis of nucleic acids) cards (Whatman Inc., Piscataway, NJ), and genomic DNA was extracted using a Purelink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA).

Identification of SNP

Primer pairs for amplification of calpastatin from genomic DNA were designed from porcine sequences as described in Lindholm-Perry et al. (2009) and those deposited in GenBank (accession number EU137105; http://www.ncbi.nlm.nih.gov/genbank/) using Primer 3 (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/primer3/input.htm). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Amplification and sequencing were performed in a Dyad PTC-0220 DNA engine (Bio-Rad, Hercules, CA) using 0.5 U of Hot Star Taq polymerase (Qiagen, Valencia, CA), 1× supplied buffer, 1.5 mM MgCl2, 200 μM deoxynucleotide 5'-triphosphate, 0.8 μM each primer, and 100 ng of genomic DNA of 12 animals from the USMARC population, representing divergent slice shear forces and sire lines in 25-μL reactions. Sequencing reactions were prepared as described in Lindholm-Perry et al. (2009).
and were sequenced on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). Polymorphisms were identified using Polyphred and assessed using Consed (http://www.phrap.org).

Table 1. Animals and phenotypes used for SNP associations

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of animals²</th>
<th>Phenotype³</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>USMARC</td>
<td>1,042</td>
<td>SSF</td>
<td>13.47 ± 3.61</td>
<td>6.4 to 31.9</td>
</tr>
<tr>
<td>Duroc-Landrace F₂</td>
<td>370</td>
<td>SSF</td>
<td>14.39 ± 4.72</td>
<td>6.79 to 33.79</td>
</tr>
<tr>
<td>OSU-NPB</td>
<td>905</td>
<td>WBSF</td>
<td>2.47 ± 0.85</td>
<td>0.88 to 7.02</td>
</tr>
<tr>
<td>Babcock</td>
<td>509</td>
<td>Star probe</td>
<td>5.71 ± 1.02</td>
<td>3.7 to 10.54</td>
</tr>
</tbody>
</table>

¹USMARC = US Meat Animal Research Center (Clay Center, NE) resource population; Duroc-Landrace F₂ = industry F₂ population (Rohrer et al., 2006); OSU-NPB = The Ohio State University (Columbus) National Pork Board industry samples; Babcock = Babcock Genetics Inc. (Rochester, MN) industry samples.

²Animals with phenotypes available.

³Slice shear force (SSF), Warner-Bratzler shear force (WBSF), and Intron Star probe (Instron, Norwood, MA) were measured in kilograms at 7 d postmortem.

Figure 1. The SNP markers genotyped in the US Meat Animal Research Center (USMARC, Clay Center, NE) resource population. A total of 131 markers were genotyped and analyzed in the USMARC resource population. The significance of associations is shown as the negative log of \( P \)-values, and the position refers to the position in GenBank accession number EU137105 (http://www.ncbi.nlm.nih.gov/genbank/) containing the complete calpastatin gene sequence. Thirty-seven markers were significantly (\( P < 0.001 \)) associated with slice shear force (see Supplemental Table 1).

SNP Genotyping

Multiplex assays for use in a Sequenom MassARRAY system (Sequenom, San Diego, CA) were designed us-
Assays were designed for approximately 30 SNP per multiplex group, and amplicon lengths were 70 to 130 bp. Reaction conditions were performed as suggested by Sequenom iPLEX chemistry.

**Association Analyses**

The data were analyzed separately for each population. Pedigreed populations were analyzed using the QTL Association option of Mendel (Lange et al., 2001), which uses the full pedigree. The statistical model included slaughter date and line as fixed effects. The proportion of phenotypic variance accounted for by the marker was calculated by \( \frac{2 \times p \times q \times \text{(effects)}^2}{\text{phenotypic variance}} \), where “effects” refers to the effect of allele substitution estimated from the regression of phenotype on number of copies of the allele.

The Ohio State University National Pork Board industry samples were analyzed with PROC MIXED (SAS Inst. Inc., Cary, NC). Loin was treated as a random effect, and regression coefficients for final cooked temperature and cooking time were fitted, along with the fixed effects of processing plant and enhancement treatment.

**Linkage Disequilibrium Analysis, and Identification of Transcription Factor Binding Sites**

Linkage disequilibrium (LD; \( r^2 \)) was estimated for the SNP using Haploview software (Barrett et al., 2005; http://www.broad.mit.edu/mpg/haploview/index.php). Haplotype blocks were based on pairwise LD values.

Transcription factor binding sites were identified in the sequence using Cister (Frith et al., 2001) as well as
Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) were performed to determine if the predicted transcription factor binding sites that contained SNP were functional and if the alternate allelic forms differed in their ability to bind nuclear proteins. Nuclear extracts (5 to 8 μg/μL) were prepared from pig skeletal muscle, heart, and testis using an NE-PER Nuclear and Cytoplasmic Extraction System (Pierce, Rockford, IL) and frozen at −80°C until use. Allele-specific oligonucleotide duplexes of 25 bp or greater were synthesized (Integrated DNA Technologies) with the polymorphic base flanked by at least 12 bp. Oligos were labeled with biotin using a 3′-end DNA Labeling Kit (Pierce) and annealed into

Table 2. Marker associations in research and industry populations1

<table>
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<tr>
<th>Marker2</th>
<th>Position3</th>
<th>USMARC</th>
<th>Duroc-Landrace F2</th>
<th>OSU-NPB</th>
<th>Babcock</th>
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<td>+Effect3</td>
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<td>13830</td>
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<td>114652</td>
<td>C (A)</td>
<td>+Effect3</td>
<td>+Effect3</td>
<td>+Effect3</td>
</tr>
</tbody>
</table>

1USMARC = US Meat Animal Research Center (Clay Center, NE) resource population; Duroc-Landrace F2 = industry F2 population (Rohrer et al., 2006); OSU-NPB = The Ohio State University (Columbus) National Pork Board industry samples; Babcock = Babcock Genetics Inc. (Rochester, MN) industry samples.
2The USMARC marker number refers to the forward sequencing primer followed by the position in contig (contiguous sequence).
4The allele with a positive effect on shear force is shown, and the allele with the negative effect is in parentheses.
5The allele with a positive effect on shear force is shown, and the allele with the negative effect is in parentheses.
6Estimated additive effect of 1 copy of the positive allele in kilograms. Empty cells indicate the marker was not genotyped or analyzed.
7Indicates the most significant markers in all 4 populations.

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duplexes. Electrophoretic mobility shift assays were performed using a Lightshift Chemiluminescent EMSA Kit (Pierce). Binding reactions containing 100 fmol of labeled probes, 0.25 to 1 µg of poly(deoxyinosinic/deoxyctydylid acid), and 10 to 12 µg of nuclear extract in the supplied buffer were incubated for 30 min on ice. Reactions were loaded on 5% native PAGE gels and electrophoresed at 100 V for 1 h on ice. Biotinylated gel products were transferred to positively charged nylon membranes and detected by chemiluminescence with a ChemiImager (Alpha Innotech, San Leandro, CA) for band detection.

**RESULTS**

**Animal Phenotypes**

The numbers of animals with slice shear force, Warner-Bratzler shear force, and Instron star probe measurements collected from 4 different populations are shown in Table 1. A total of 2,826 phenotyped animals in 4 populations were used in this study.

**Resequencing and SNP Discovery**

We sequenced about one-third of the calpastatin gene (more than 38,000 bp) in 12 animals from the USMARC resource population representing the most tender and tough slice shear force values. These animals also differed in their genotypes at the most predictive markers previously identified for slice shear force (Lindholm-Perry et al., 2009). This sequence includes the complete coding and untranslated regions and all the predicted mRNA splice sites and regulatory (promoter) regions. A total of 194 SNP and 22 insertions/deletions (indels) were identified in this sequence. These polymorphisms were targeted for genotyping on a Sequenom MassArray system. A larger indel (77003_101–116) was genotyped on polyacrylamide gels.

**Genotyping and Association with Tenderness**

Assay design was attempted for 184 SNP. Ten SNP were intractable because of their repetitive nature and their position in the sequence; these genotypes could be inferred from other SNP in the research population. Additionally, an indel with 3 alleles (77003_101–116) was genotyped by fragment size on polyacrylamide gels using infrared-labeled primers (Li-Cor, Lincoln, NE). Of these SNP, 18 were monomorphic (sequence artifacts or assay failure), 6 were completely heterozygous (sequence differences in repetitive elements), and 29 would not amplify or give reliable genotypes. An additional 9 markers from a previous study were included (Lindholm-Perry et al., 2009). In total, 131 markers were analyzed in the USMARC resource population (Figure 1), and details are given in Supplemental Table 1. Thirty-seven markers were significantly \((P < 0.001)\) associated with slice shear force in the USMARC popu-
lation. These markers and 3 others that were previously significant in the resource population were assayed in the other 3 populations (Figure 2 and Table 2). Four of these markers were highly significant in all 4 populations (67853_270, 67855_230, 67855_289, and 77013_98; Table 2) and accounted for about 1% of the phenotypic variance. These markers were also associated in differing degrees with sensory panel measurements of juiciness, chewiness, tenderness, connective tissue, and flavor scores (Table 3). When significant, the favorable, tender allele for instrumental measurements was consistently associated with the favorable sensory panel score.

### Associations with Meat Quality and Other Traits

Some of the markers most significantly associated with tenderness were also associated with cooking loss and purge in the 4 populations (Table 4). When significant, the favorable, tender allele for instrumental measurements was associated with a favorable meat quality score. Some of these markers were also associated with fatness traits, such as marbling and intramuscular fat (Table 5; $P < 0.01$). No markers were associated with BW gain from 8 to 22 wk of age or backfat (data not shown).

### Table 4. The SNP marker associations with meat quality and other traits

<table>
<thead>
<tr>
<th>Marker</th>
<th>Babcock Cooking loss</th>
<th>Babcock Purge</th>
<th>OSU-NPB Cooking loss</th>
<th>USMARC Cooking loss</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Chop purge</td>
<td>Loin purge</td>
<td>Chop purge</td>
<td>Loin purge</td>
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<td>0.08408</td>
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<td>0.00016</td>
</tr>
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</table>

1Babcock = Babcock Genetics Inc. (Rochester, MN) industry samples; Duroc-Landrace F2 = industry F2 population (Rohrer et al., 2006); OSU-NPB = The Ohio State University (Columbus) National Pork Board industry samples; USMARC = US Meat Animal Research Center (Clay Center, NE) resource population.

2Estimated effects of 1 copy of the positive allele are shown in the first row, and $P$-values are shown below for each marker and trait. The favorable allele coincides with the favorable allele for tenderness.

### Table 5. The SNP marker associations with fatness traits

<table>
<thead>
<tr>
<th>Marker</th>
<th>Duroc-Landrace F2 Fat %</th>
<th>Marbling</th>
<th>Image analysis fat</th>
<th>Intramuscular fat</th>
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<tbody>
<tr>
<td>67831_430</td>
<td>0.5743</td>
<td>0.0225</td>
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<td>0.22207</td>
<td>0.45550</td>
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<td>0.00255</td>
<td>0.07927</td>
<td>0.01649</td>
<td>0.00839</td>
</tr>
</tbody>
</table>

1Duroc-Landrace F2 = industry F2 population (Rohrer et al., 2006); OSU-NPB = The Ohio State University (Columbus) National Pork Board industry samples.

2Estimated effects of 1 copy of the positive allele are shown in the first row, and $P$-values are shown below for each marker and trait. The favorable allele coincides with the favorable allele for tenderness.
Promoter Predictions

A total of 198 transcription factor binding sites were predicted in the calpastatin sequence (Supplemental Table 2). These sites tended to be clustered in the previously reported promoter regions (Meyers and Beever, 2008). Thirty-one SNP were found in transcription factor binding sites, and alternate alleles are predicted to alter or obliterate transcription factor binding (Supplemental Table 2).

Allele Frequencies

The allele frequencies of the SNP most significantly associated with instrumental measures of tenderness in the different populations sampled were similar. These are shown in Table 6.

LD

The LD patterns were similar in the different populations, and several of the significant SNP were in high LD with each other (Supplemental Figure 1). When analyzing the 40 SNP assayed in all populations, the SNP fell into 5 haplotype blocks. The first block contained SNP 67831_429 and 67831_430, which were in complete LD with each other, block 2 contained SNP 66602_392, 67853_270, 67855_230, and 67855_289, and block 3 contained 67857_306 and 77013_98 (Supplemental Figure 1).

EMSA

Electrophoretic mobility shift assays were designed for 16 polymorphic sites. The sites were chosen by the probability of transcription factor binding and their association with tenderness in the resource population (Supplemental Table 2). Six sites demonstrated a gel shift when probes were incubated with nuclear extract from muscle, heart, or testis. Four of these sites, a specificity protein 1 (Sp1) site around nucleotides 12978 and 12979 (67831_429/430) of the calpastatin gene, a potential thyrotroph embryonic factor (Tef) site around nucleotide 25587 (67841_556), an unpredicted site around nucleotide 48699 (67855_230), and myocyte enhancer factor-2 (Mef-2)/TATA sites around positions 49223 (67857_306) and 49228 (77013_98) were allele specific in binding nuclear proteins (Figure 3). Competition of nuclear protein binding was demonstrated using 100-fold excess of the unlabeled allele-specific probe.

DISCUSSION

Consumer perception of pork eating quality is greatly influenced by tenderness (Moeller et al., 2010). Unfortunately, recent intense selection for increased lean growth has resulted in reduced meat quality and issues with consumer acceptance (Schwab et al., 2006). Although pork quality is not directly an economically important trait, demand is ultimately determined by consumer preference, and selection for pork quality would increase consumer acceptance and benefit the pork industry.

This study identified a panel of SNP in regulatory regions of the calpastatin gene that were consistently associated with different measures of tenderness (i.e., slice shear force, Warner-Bratzler shear force, Instron force, and sensory taste panel scores) in 4 separate industry-relevant populations. The allele frequencies of these SNP in crossbred populations imply that significant improvement for tenderness could be made by genetic selection of the tender allele. These markers were also favorably associated with other pork quality traits, with the tender allele being associated with less purge and cooking loss and with greater marbling and intramuscular fat. Pork quality traits were found to be correlated, and sensory and instrumental measures of tenderness were found to be correlated with cook loss, drip loss, marbling, and color (Huff-Lonergan et al., 2002). The markers were not associated with BW gain from 8 to 22 wk or backfat (data not shown), nor were they negatively associated with any meat quality traits tested. Therefore, use of these markers for selection should not negatively affect these other economically important traits. The most significant SNP associations were found in the calpastatin muscle-specific transcript type III promoter preceding exon 1u (Sensky et al., 2008).

Table 6. Allele frequencies of the most significant SNP in sampled populations

<table>
<thead>
<tr>
<th>Marker</th>
<th>Positive allele</th>
<th>USMARC</th>
<th>Duroc-Landrace F2</th>
<th>OSU-NPB</th>
<th>Babcock</th>
</tr>
</thead>
<tbody>
<tr>
<td>67831_429/430</td>
<td>A/G</td>
<td>0.195</td>
<td>0.220</td>
<td>0.192</td>
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</tr>
<tr>
<td>66602_392</td>
<td>C</td>
<td>0.641</td>
<td>0.894</td>
<td>0.644</td>
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</tr>
<tr>
<td>67853_270</td>
<td>A</td>
<td>0.709</td>
<td>0.892</td>
<td>0.641</td>
<td>0.819</td>
</tr>
<tr>
<td>67855_230</td>
<td>G</td>
<td>0.715</td>
<td>0.882</td>
<td>0.639</td>
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</tr>
<tr>
<td>67855_289</td>
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<td>0.727</td>
<td>0.893</td>
<td>0.648</td>
<td>0.769</td>
</tr>
<tr>
<td>67857_306</td>
<td>G</td>
<td>0.506</td>
<td>0.310</td>
<td>0.392</td>
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<tr>
<td>77013_98</td>
<td>G</td>
<td>0.724</td>
<td>0.842</td>
<td>0.639</td>
<td>0.809</td>
</tr>
</tbody>
</table>

1USMARC = US Meat Animal Research Center (Clay Center, NE) resource population; Duroc-Landrace F2 = industry F2 population (Rohrer et al., 2006); OSU-NPB = The Ohio State University (Columbus) National Pork Board industry samples; Babcock = Babcock Genetics Inc. (Rochester, MN) industry samples.
2The positive allele is associated with greater instrumental force.
Meyers and Beever (2008) found that heterozygosity in the first 60 kb of calpastatin was concordant with the QTL for tenderness in their Illinois Meat Quality Pedigree population, suggesting that variation affecting tenderness resides in an upstream regulatory region of calpastatin. The SNP most significantly associated with tenderness in this study were located within the first 50 kb of the calpastatin gene. These SNP were more significantly associated with shear force and Instron force than were 3 SNP reported previously that change AA (41646_874, Ser/Asn; 41650_892, Lys/Arg; and M20160_638, Ser/Arg; Ciobanu et al., 2004). These SNP reside in predicted transcription factor binding sites, and allelic forms are predicted to differ in binding strength based on the probability of the consensus sequence (Frith et al., 2001). This prediction was supported by gel mobility shift assays, where the allele with the greater probability and associated with tougher meat was more effective in binding nuclear proteins. These SNP at the sites that bound nuclear proteins were not in complete LD with each other and may independently affect calpastatin expression.

The identification of predictive markers in calpastatin will allow producers to benefit from marker-assisted selection to improve pork tenderness, and possibly to benefit from marker-assisted management and slaughter strategies to produce a more desirable product.

**LITERATURE CITED**


