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A Limousin specific myostatin allele affects longissimus muscle area and fatty acid profiles in a Wagyu-Limousin F_2 population[1,2]

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ABSTRACT: A microsatellite-based genome scan of a Wagyu × Limousin F_2 cross population previously demonstrated QTL affecting LM area and fatty acid composition were present in regions near the centromere of BTA2. In this study, we used 70 SNP markers to examine the centromeric 24 megabases (Mb) of BTA2, including the Limousin-specific *F94L* myostatin allele (AB076403.1; 415C > A) located at approximately 6 Mb on the draft genome sequence of BTA2. A significant effect of the *F94L* marker was observed ($F = 60.17$) for LM area, which indicated that myostatin is most likely responsible for the effect. This is consistent with previous reports that the substitution of Leu for Phe at AA 94 of myostatin (caused by the 415C > A transversion) is associated with increased muscle growth. Surprisingly, several fatty acid trait QTL, which affected the amount of unsaturated fats, also mapped to or very near the myostatin marker, including the ratio of C16:1 MUFA to C16:0 saturated fat ($F = 16.72$), C18:1 to C18:0 ($F = 18.88$), and total content of MUFA ($F = 17.12$). In addition, QTL for extent of marbling ($F = 14.73$) approached significance ($P = 0.05$), and CLA concentration ($F = 9.22$) was marginally significant ($P = 0.18$). We also observed associations of SNP located at 16.3 Mb with KPH ($F = 15.00$) and for the amount of SFA ($F = 12.01$). These results provide insight into genetic differences between the Wagyu and Limousin breeds and may lead to a better tasting and healthier product for consumers through improved selection for lipid content of beef.

Key words: carcass, cattle, Limousin, myostatin, quantitative trait locus, Wagyu

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INTRODUCTION

Reports in the popular press that dietary SFA may have adverse health effects has led consumers to be more conscious of SFA in their diet. Relative to other breeds, Wagyu beef has more marbling and an increased ratio of MUFA fat to SFA (May et al., 1993; Elias Calles et al., 2000). This increased ratio has human health implications in terms of overall health and decreasing coronary heart disease (McDonald, 1991). Experimental comparisons of Limousin and Wagyu germplasm indicate decisive breed differences with respect to carcass attributes (Kuber et al., 2004; Pitchford et al., 2006). Wagyu cattle are smaller framed, have more marbling, and take considerably longer to finish in a feedlot than Limousin, making them less desirable from a production standpoint.

In previous studies (Alexander et al., 2007a,b; Tshipuliso et al., 2008), we identified 7 QTL on 5 chromosomes involved in carcass traits and lipid metabolism. A QTL for LM area (**LMA**) and a QTL with multifaceted effects on CLA, marbling, MUFA, SFA, $\mathbf{R_2}$ (ratio of C16:1 to C16:0), $\mathbf{R_3}$ (ratio of C18:1 to C18:0), and having a small effect on flavor were observed toward the centromere of BTA2 in which region *Myostatin* (***MSTN***) resides. Myostatin, also known as growth differentiation factor 8 (***GDF8***), has been previously associated with muscular hypertrophy, also known as

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[2]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.

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double muscling (Kambadur et al., 1997; Grobet et al., 1998).

In this study we used 70 SNP covering the centromeric 24 megabases (**Mb**) of BTA2 to fine map the fatty acid QTL. These markers included a SNP for *F94L* at the *MSTN* gene (Grobet et al., 1997; Dunner et al., 2003). Somewhat surprisingly, it appears that myostatin, or a separate gene in close proximity, is exhibiting a pleotropic effect on quantity and composition of fat, as well as on LMA. Application of the results presented here may lead to a better tasting and healthier product for consumers and provide marker information for producers for breeding purposes.

MATERIALS AND METHODS

The animals used in the study were cared for according to the guidelines of the USDA-ARS Fort Keogh Livestock and Range Research Laboratory Institutional Animal Care and Use Committee.

Animals and Phenotypes

Descriptions of animals and phenotypes have been reported previously (Alexander et al., 2007a,b). Briefly, the pedigree consisted of 8 Wagyu bulls mated to 108 Limousin females to produce 121 F_1 females over a 3-yr period. Three of the 8 Wagyu bulls also sired 6 F_1 bulls. The F_1 offspring were mated inter se, except that mating of known relatives was avoided. These matings produced 328 F_2 progeny (303 with phenotypes in this study) between 2000 and 2003. Phenotypes included taste panel-scored flavor; R_2; R_3; CLA; and KPH, marbling, MUFA, and SFA and are described in Alexander et al. (2007a,b).

Selection of SNP

From the collection of various putative bovine SNP sources (for a summary, see van Tassell et al., 2008), 4,125 SNP were selected that mapped to the centromeric 24-Mb region of BTA2. Of these, 101 SNP were chosen for uniform spacing to represent the whole region, and mass-spectroscopy assays (Sequenom, San Diego CA) were designed. The description of individual sources of SNP is given in Supplemental Table 1 (http://jas.fass.org/content/vol87/issue5). The spacing and gap distribution of the SNP chosen for this study are given in Supplemental Figure 1 (http://jas.fass.org/content/vol87/issue5).

Genotyping of SNP

The C to A transition mutation causing the F94L substitution in the myostatin protein was genotyped by use of a homogeneous mass extension MassARRAY assay as recommended by the manufacturer (Sequenom). The forward amplification primer for the assay was 5′-AATGAGAACAGCGAGCAG-3′ and the reverse amplification primer was 5′-GCGTGGTAGTCATCGTC-3′. Both amplification primers had additional nontemplate bases on the 5′ ends to make the total primer length 30 bases as per the homogeneous mass extension MassARRAY protocol. The probe extension primer was designed to anneal to the upper strand (extension toward the 5′ end of the gene) with sequence 5′-GCTGGCATCTCTCTGGACATC-3′. Extension products from this probe added either a thymidine (T), representing the A allele (coding for the Limousin-specific Leu), or a guanosine (G), representing the C allele (producing the normal Phe). The other SNP were genotyped using MassArray iPLEX assays as recommended by the manufacturer (Sequenom). The amplification primers for each assay are given in Supplemental Table 2 (http://jas.fass.org/content/vol87/issue5). Genotypes that produced "conservative" calls on the instrument were assumed to be correct, except where they were not consistent between 2 replicates or where they violated Mendelian expectations. Inconsistent calls and genotypes flagged as "moderate" or "aggressive" by the SpectroTyper software (Sequenom) were evaluated by hand and corrected where necessary.

Analyses of SNP

Markers that failed to produce genotypes, that were monomorphic in the population, or that produced all heterozygous genotypes were eliminated from consideration. In addition, a multilocus version of GenoProb (Thallman, 2002) was used to screen SNP for non-Mendelian inheritance. Of the 101 original SNP, 70 were retained for association analyses. Markers were fitted as additive covariates (0, 1, 2 copies) in separate models that included a random polygenic effect to account for genetic similarities attributable to family structure (Allan et al., 2007; Kuehn et al., 2007). Fixed effects included sex and year, with age at sampling as a linear covariate. Variance component estimates for the random polygenic effect and residual behaved erratically, likely because of the limited number of animals. Therefore, the variance of the polygenic effect was fixed at 40% of the phenotypic variance. This ratio reflects the moderate to great heritability associated with most carcass measures. All tests for associations were conducted using MTDFREML (Boldman et al., 1995). A Bonferroni correction factor was applied to the resulting associations based on 70 SNP tests; an F-value of 11.7 was required for the association to be significant at the $\alpha = 0.05$ level. For one trait (LMA), the most significant marker (*F94L*) was fitted simultaneously within models with each other marker to determine whether other SNP were significant because of their correlation with *F94L*.

Generation of Myostatin Sequences

The myostatin gene sequence was retrieved from the National Center for Biotechnology Informa-

Table 1. Primers used to amplify growth differentiation factor 8 (GDF8) exons

Primer name	Exon	5′ to 3′ primer sequence	Amplicon size, bp
BTMYOE1F	1	cga gat tca ttg tgg agc aa	730
BTMYOE1R	1	gct ctt tcc cct cct cct ta	
BTMYOE2F	2	ttt tgc tgt tat gaa tga aat gc	654
BTMYOE2R	2	agg tgc ctt tgt ctg gct ta	
BTMYOE3AF	3	gat ctg acc aca ggg gaa tc	592
BTMYOE3AR	3	tga aca ccc aca gcg atc ta	
BTMYOE3BF	3	agg cca att act gct ctg ga	522
BTMYOE3BR	3	cca gaa gac aag gag aat tgc	
BTMYOE3CF	3	atg cag gtg aat gaa agc aa	504
BTMYOE3CR	3	ttt gac tgc ctt ttc aat gc	
BTMYOE3DF	3	ctg cat tga aaa ggc agt ca	839
BTMYOE3DR	3	cca aac ttt tgt gct cag tca	

tion nucleotide database under accession number gi|76609309|ref|NW_930818.1|Bt2_WGA288_2 *Bos taurus* chromosome 2 genomic contig. Primer 3 software (Rozen and Skaletsky, 2000; at http://fokker.wi.mit.edu/primer3/input.htm) was used to create primers for amplification and sequencing of the 3 exons of myostatin. Primers were designed flanking exons 1 and 2. A set of 4 overlapping primers were used to amplify exon 3. The 6 PCR primer pair sequences and amplicon sizes are given in Table 1. Standard PCR protocols were performed. Briefly, samples were denatured for 5 min at 95°C, followed by 36 cycles of 95°C for 30 s, 57°C for 75 s, and 72°C for 75 s, with a final extension at 72°C for 10 min. Template was stored at 4°C. Amplicons were purified for sequencing by using Exosapit (Amersham, Piscataway, NJ) and sequenced by using ABI Big Dye chemistry on an ABI 3730 instrument (ABI, Foster City, CA). The SNP were detected using Sequencher software (Gene Codes, Ann Arbor, MI).

RESULTS

Initially, 101 markers (Supplemental Table 2; http://jas.fass.org/content/vol87/issue5) covering the 24-Mb centromeric region of BTA2 were assayed, with 70 yielding satisfactory genotypic data. Markers were considered unsatisfactory if they were monomorphic or did not follow Mendelian inheritance. The spacing, gap distribution, and minor allele frequencies of the markers are shown in Supplemental Figure 1 (http://jas.fass.org/content/vol87/issue5). A total of 460 animals were genotyped in this study. Four marker assays worked in fewer than 300 animals, and a total of 20 assays gave calls for fewer than 400 animals. Among the 95 assays that worked, the average number of animals with calls was 420. From a sample quality perspective, 8 animals worked for fewer than half of the assays, probably indicative of a DNA problem. The average number of assays that worked per animal was 86, with 235 animals that gave genotypes for >90 assays, but none that gave genotypes for all 95 markers. Seventeen of the assays were monomorphic. The targeted F94L assay worked very well in most animals.

Resulting *F*-statistics relative to physical map positions are plotted in Figure 1, which is a simplified version of Supplemental Figure 2 (http://jas.fass.org/content/vol87/issue5) for each trait of interest. No significant associations were detected for CLA or flavor. Only single markers were significant for KPH and SFA (*BTB-01112778* for both traits) and for marbling (*BTA-00173-rs29013334*). For MUFA, R_3, R_2, and LMA traits, 3, 3, 5, and 20 markers were significant, respectively, across the examined region. Table 2 summarizes the SNP that were most significant for each of these traits, as well as their founder frequencies in each base breed. Across all 7 traits, 4 markers were most significant, with *F94L* having the greatest *F*-statistic for 3 traits (LMA, MUFA, R_3). Correlations among the additive effects of these 4 markers were moderate (−0.613 to 0.612).

The *F94L* marker was significant for several traits. Our data show that the effect of increased LMA came from the L94 Limousin allele, whereas the increased MUFA and R_3 came from the F94 Wagyu allele. All marker associations with LMA were retested in a model that included *F94L*. In this analysis, all markers were nonsignificant, with the exception of *BTB-0007898*; its *F*-value was 13.3 (originally 42.5 when fitted without *F94L)*.

The result that the *F94L* marker in myostatin was associated with several traits led us to sequence the entire coding region of the *MSTN* gene to determine if other mutations were segregating in the populations. The sequence of 6 F_1 bulls and 2 of the founder bulls for which we had DNA revealed 11 total SNP in the derived sequences of the *GDF8* gene (Table 3). Seven of the SNP were in the 3′ untranslated region, 2 were in the first exon, 1 was in the first intron, and 1 was in the second exon. The *F94L* was 1 of the SNP in the first exon, whereas the other was silent. The SNP in the second exon was found in only 1 of the founder Wagyu bulls and was not present in any of the 6 F_1 bulls. This base change was AB076403.1; 2345C > A, which resulted in a Ser to Thr change at AA 125. Because of the conservative nature of this change, we did not genotype for this SNP.

DISCUSSION

Associations detected from these marker tests confirm the QTL previously detected in this population in a whole-genome scan using microsatellite markers (Alexander et al., 2007a,b; Tshipuliso et al., 2008). In these previous studies, we identified 7 QTL on 5 chromosomes involved in carcass traits and lipid metabolism by using interval mapping (Seaton et al., 2002). A QTL for LMA ($F = 45.39$) and a QTL with multifaceted effects on CLA ($F = 12.01$), marbling ($F = 11.76$), MUFA ($F = 21.37$), SFA ($F = 12.52$), R_2 ($F = 19.44$), R_3 ($F = 25.18$), and having a small effect on flavor ($F = 7.91$) were observed toward the centromere of BTA2.

Quantitative trait loci that affected LMA have been described previously by Casas et al. (1998), Sellick et al. (2007), Alexander et al. (2007a), and Abe et al. (2008) at the centromeric end of BTA2 near *MSTN*. The myostatin locus (*GDF8*) has previously been associated with muscular hypertrophy (Kambadur et al., 1997; Grobet et al., 1998). Numerous mutations in *MSTN* have been characterized in many breeds (Casas et al., 1998; Grobet et al., 1998; Dunner et al., 2003). The mutations vary from an 11-bp deletion in the heavily double-muscled Belgian Blue cattle (Grobet et al., 1997), which causes truncation of the bioactive carboxy terminus, to a C to A change at position 282 in the first exon (Grobet et al., 1998), an AA change of Phe to Leu (*F94L*), where the double-muscle phenotype is not as pronounced. Grobet et al. (1998) and Dunner et al. (2003) showed that Limousins were at an increased frequency for the F94L substitution when compared with Holsteins. However, Grobet et al. (1998) described this substitution as a conservative AA change and subsequently discounted the Limousin allele as playing a role in muscular hypertrophy. We previously questioned (Alexander et al., 2007a) the conclusion of Grobet et al. (1998) that a hydrophobic aromatic AA (Phe) replaced by an aliphatic AA (Leu) is conservative. Based on data reported here, we conclude that the *F94L* mutation does have a role in carcass size, at least affecting the LM in a Wagyu background. These results, relative to LMA, have recently been confirmed by Esmailizadeh et al. (2008).

As stated above, the *F94L* marker was significant for several traits. Our data show that the effect of increased LMA came from the L94 Limousin allele, whereas the increased MUFA and R_3 arose from the F94 Wagyu allele. Haplotyping was attempted in this region to identify whether effects were due to F94L or other potential haplotypes. However, essentially only 1 haplotype was found with the L94 Limousin allele over a 2-Mb region, suggesting a pleotropic effect for GDF8, whereas 8 to 10 haplotypes were identified containing the F94 Wagyu, preventing a further analysis because of too few animals representing each haplotype. Figure 1 shows multiple significant peaks for LMA, with peaks for other traits lying underneath these LMA peaks. Although it is possible that there are multiple QTL, each

Table 2. Single nucleotide polymorphisms with the most significant association detected for each meat quality trait

Trait[1]	SNP	*F*-statistic (*P*-value)[2]	Location, Mb	Alleles A0/A1	Limousin A0 frequency	Wagyu A0 frequency	F2 A0 frequency	Additive effect ±S, A0 → A1
LMA	F94L	60.17 (9.6×10^{-9})	6.533	C/A	0.10	1.00	0.55	6.76 ± 0.87
KPH	BTB-01112778	15.00 (0.009)	19.822	A/G	0.17	0.88	0.56	−0.110 ± 0.041
Marbling	BTA-00173-rs29013334	14.73 (0.011)	3.921	A/G	0.95	0.43	0.60	0.407 ± 0.106
MUFA	F94L	17.12 (0.003)	6.533	C/A	0.10	1.00	0.55	−1116.1 ± 269.7
SFA	BTB-01112778	12.01 (0.042)	19.822	A/G	0.17	0.88	0.56	−710.1 ± 204.9
R_2	BFGL-INS-57624	16.72 (0.004)	6.401	A/G	0.96	0.75	0.79	0.844 ± 0.207
R_3	F94L	18.88 (0.001)	6.533	C/A	0.10	1.00	0.55	−19.3 ± 4.4

[1]LMA = LM area (cm^2); KPH (%); amount of MUFA (mg/100 g of dry tissue); amount of SFA (mg/100 g of dry tissue); R_2 = ratio of C16:1 to C16:0 (%); R_3 = ratio of C18:1 to C18:0 (%).
[2]*P*-value calculated using Bonferroni.

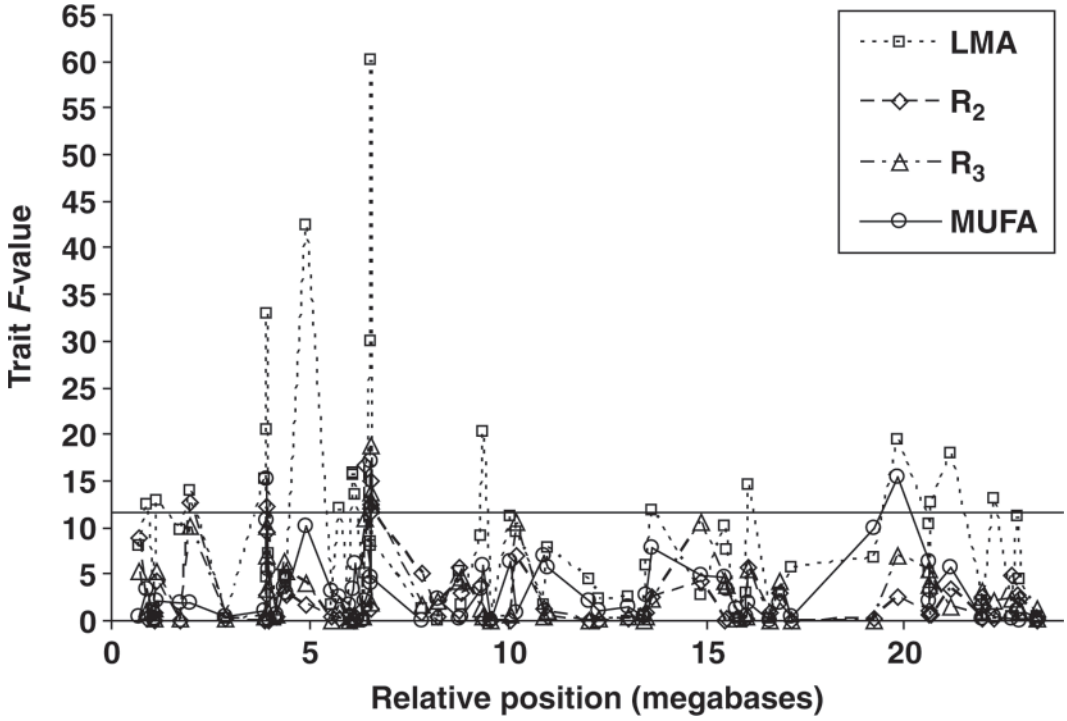

Figure 1. *Bos taurus* chromosome 2 *F*-statistic profiles for LM area (LMA), ratio of C16:1 to C16:0 fatty acids (R_2), ratio of C18:1 to C18:0 fatty acids (R_3), and MUFA. The genome-wide significance threshold of 11.7 is shown as a horizontal line. Bovine genome assembly 4.0 was used.

having a pleotropic effect on the traits examined, a more likely explanation for this result is problems in the presumed order of the markers (we used bovine genome assembly version 4.0 for marker positioning). Future genome assemblies for this region may indicate fewer peaks for these traits.

The role of the Wagyu myostatin allele(s) on the fatty acid profile remains unclear. If this allele is fixed (Phe) in the Wagyu line and nearly fixed for Leu in the Limousin line, then the marker approximates a test of breed-of-origin differences similar to the initial scans in Alexander et al. (2007a,b). The significance of the *F*-tests for the associations in this study for both MUFA and R_3 was slightly less than the tests reported in Alexander et al. (2007b). Therefore, the *F94L* marker may only be optimal in this study for these fatty acid traits. This result is in stark contrast to the significance for the LMA effect detected for *F94L* in this study, which was much more significant than the QTL reported in Alexander et al. (2007a). However, *F94L* remains the most likely candidate for fatty acid differences from this study. Because we did not detect any major SNP

Table 3. Single nucleotide polymorphisms detected in 2 founder Wagyu bulls and 6 F_1 bulls used in this project

Bull	400[1]	415	2290	2345	5283	5291	5312	5333	5395	5405	6173
914077	A/A	C/C	C/T	C/C	A/C	T/A	A/C	A/C	C/T	A/G	A/C
914400	G/G	C/C	C/C	A/C	C/C	T/T	C/C	A/A	T/T	A/G	A/A
981470	A/A	C/C	C/T	C/C	A/C	T/A	A/C	A/C	C/T	A/G	ND[2]
981471	A/A	C/A	C/T	C/C	A/C	T/A	A/C	A/C	C/T	A/G	A/C
981472	A/A	C/A	T/T	C/C	A/A	T/T	A/A	C/C	C/C	G/G	C/C
981473	A/A	C/A	C/T	C/C	A/C	T/A	A/C	A/C	C/T	A/G	A/C
981474	A/A	C/A	T/T	C/C	A/A	T/T	A/A	C/C	C/C	G/G	C/C
981475	A/G	C/A	C/T	C/C	A/C	T/A	A/C	A/C	C/T	G/G	A/C
	1st exon[3]	1st exon	1st intron	2nd exon	3′ UTR[4]	3′ UTR	3′ UTR	3′ UTR	3′ UTR	3′ UTR	3′ UTR
AA change	None	F94L	NA[5]	S125T	NA	NA	NA	NA	NA	NA	NA
Wagyu[6]	A	C	T	C	C	A	C	A	T	A	A

[1]Base pair position of the SNP when compared with the Wagyu *GDF8* gene (AB076403.1).
[2]ND = not determined.
[3]Location of the SNP in the gene structure.
[4]UTR = untranslated region.
[5]NA = not applicable
[6]Nucleotide found in AB076403.1.

in the F_1 bull *GDF8* sequences that were different from other breeds, we cannot propose a mechanism regarding how or why the fatty acid profiles differ. How these differences occur will be the focus of future research.

As stated in the results, correlations among the most significant markers were only moderate, implying that linkage disequilibrium was playing a minimal role in the associations detected across this region. One possible exception may be in the marker association tests for LMA, primarily because of the increased level of significance associated with this marker. Other markers needed only a moderate correlation with *F94L* to be significant for LMA when each marker was tested individually. Hence, most marker associations for LMA were no longer significant when tested in a model that included *F94L*. This result suggests that, although other markers in the region (as evidenced by *BTB-0007898* remaining significant) may have an effect on LMA, *F94L* still seems to be a strong candidate for causal variation. Our data suggest that the effects of variation in myostatin expression or function may have an impact beyond muscle growth, altering fat abundance and composition.

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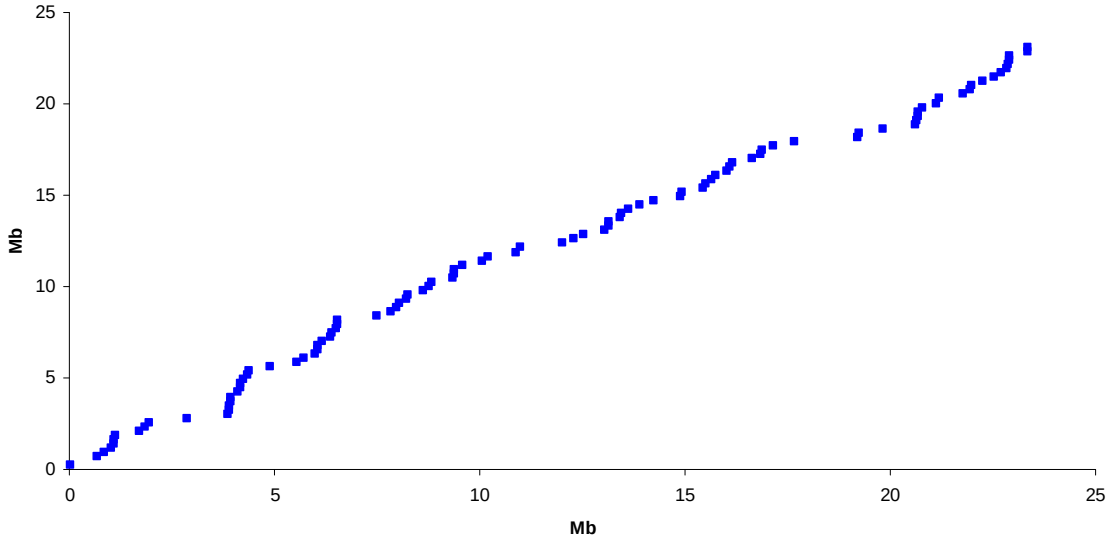
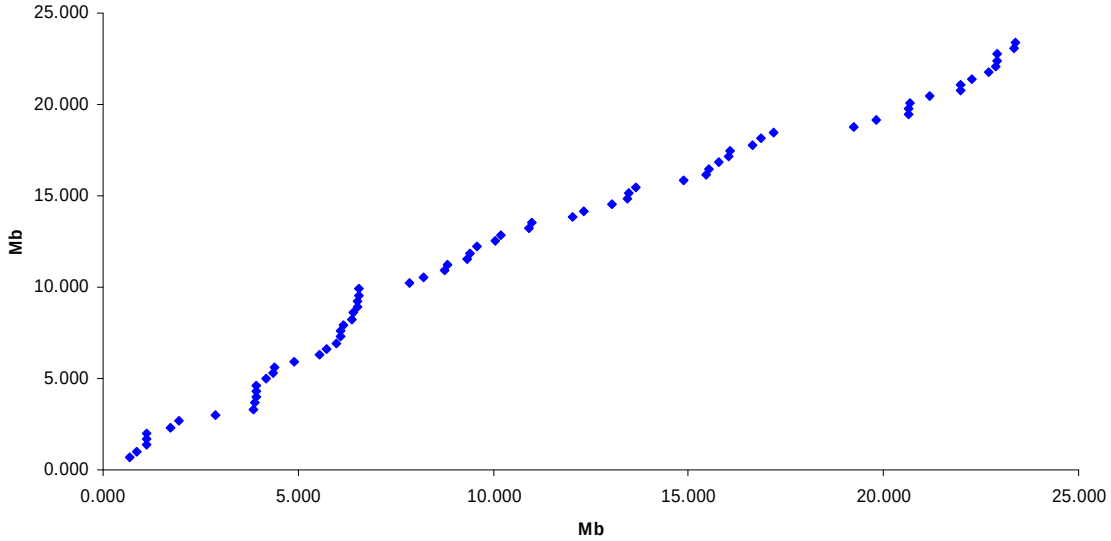
Supplemental Figure 1.
a) Singe nucleotide polymorphism distribution across bovine chromosome 2 for all genotyped markers.



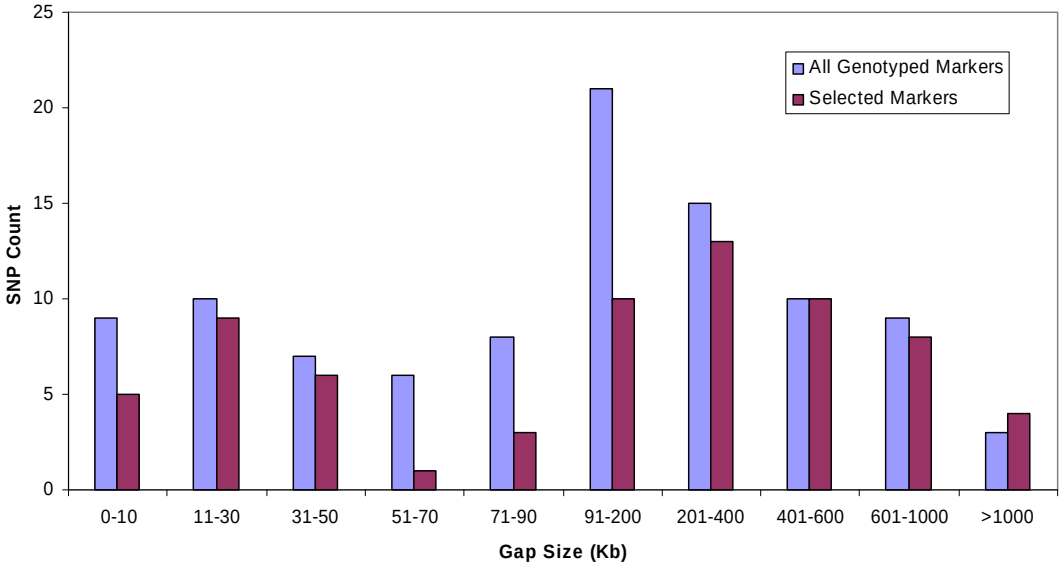
In the scatter plot, the X-coordinate is the actual position in megabases (Mb) on Btau_4.0, whereas the Y-coordinate is the calculated position assuming equal spacing between the 101 markers initially screened. Locations of large gaps and closely spaced SNP can be visualized.

b) Singe nucleotide polymorphism distribution of selected markers.

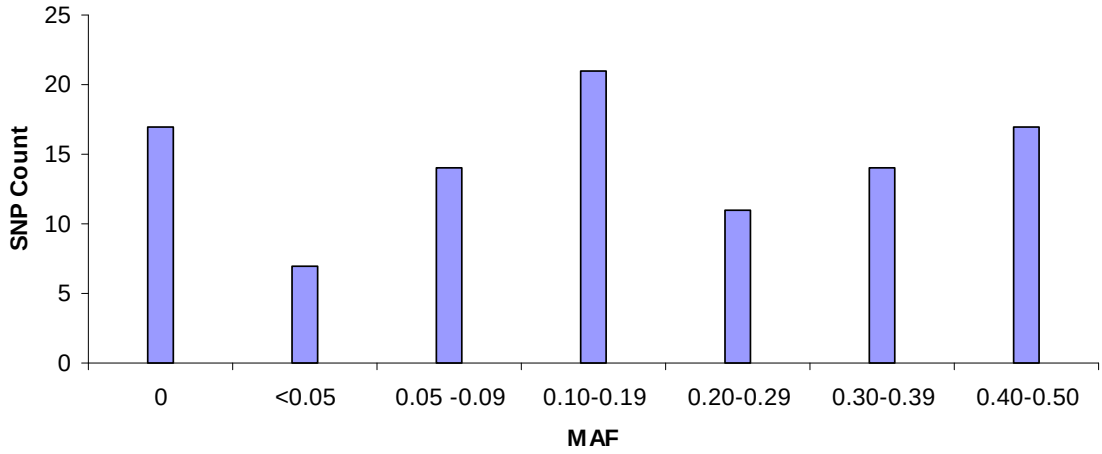


In the scatter plot the X-coordinate is the actual position in Mb on Btau_4.0, whereas the Y-coordinate is the calculated position assuming equal spacing between the 70 markers that were selected for the SNP association analysis.

c) Gap distribution of adjacent markers genotyped on the assay.



d) Minor allele frequencies of genotyped SNP markers.



Supplemental Figure 2. *Bos taurus* chromosome 2 *F*-statistic profiles for LM area (LMA), ratio of C16:1 to C16:0 fatty acids (R_2), ratio of C18:1 to C18:0 fatty acids (R_3), marbling score, SFA, percent KPH, CLA, and flavor. The genome-wide significance threshold of 11.7 is shown as a horizontal line.