

Candidate gene analysis of *GH1* for effects on growth and carcass composition of cattle

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Summary

We present an approach to evaluate the support for candidate genes as quantitative trait loci (QTLs) within the context of genome-wide map-based cloning strategies. To establish candidacy, a bacterial artificial chromosome (BAC) clone containing a putative candidate gene is physically assigned to an anchored linkage map to localise the gene relative to an identified QTL effect. Microsatellite loci derived from BAC clones containing an established candidate gene are integrated into the linkage map facilitating the evaluation by interval analysis of the statistical support for QTL identity. Permutation analysis is employed to determine experiment-wise statistical support. The approach is illustrated for the growth hormone 1 (*GH1*) gene and growth and carcass phenotypes in cattle. Polymerase chain reaction (PCR) primers which amplify a 441 bp fragment of *GH1* were used to systematically screen a bovine BAC library comprising 60 000 clones and with a 95% probability of containing a single copy sequence. The presence of *GH1* in BAC110R2C3 was confirmed by sequence analysis of the PCR product from this clone and by the physical assignment of BAC110R2C3 to bovine chromosome 19 (BTA19) band 22 by fluorescence *in situ* hybridisation (FISH). Microsatellite *KHGH1* was isolated from BAC110R2C3 and scored in 529 reciprocal backcross and F₂ fullsib progeny from 41 resource families derived from Angus (*Bos taurus*) and Brahman (*Bos indicus*). The microsatellite *KHGH1* was incorporated into a framework genetic map of BTA19 comprising 12 microsatellite loci, the erythrocyte antigen T and a *GH1-TaqI* restriction fragment length polymorphism (RFLP). Interval analysis localised effects of *taurus* vs. *indicus* alleles on subcutaneous fat and the percentage of ether extractable fat from the *longissimus dorsi* muscle to the region of BTA19 harbouring *GH1*.

Keywords: bovine, BTA19, genetic linkage, *GH1*, interval analysis, permutation test

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Introduction

Interest in the localisation of quantitative trait loci (QTLs) in beef cattle that influence variation in carcass composition (e.g. fat deposition sites and lean tissue yield) and quality (e.g. muscle tenderness and palatability) stems from the importance of these characteristics to the determination of carcass value and consumer satisfaction. However, many of these characteristics cannot be measured in live animals, and therefore, they are recalcitrant to improvement through the application of within breed selection programmes. In contrast, live weight at slaughter is a major determinant of carcass value which is highly heritable, possesses significant phenotypic variation and is highly responsive to directional selection within beef cattle. Unfortunately, weights at all ages are positively genetically and phenotypically correlated and selection for increased weight at maturity usually results in an increase in birth weight and concomitant losses in productivity due to calving difficulty. However, the existence of developmentally regulated genes influencing growth in mice is well known (Cheverud *et al.* 1996) and suggests that it may be possible to identify QTLs in cattle that significantly influence mature weight without having a concomitant effect on birth weight. Beever *et al.* (1990) localised QTLs influencing 205 day and 365 day weights, preweaning average daily gain and subcutaneous fat thickness, but not birth weight, to the region of bovine chromosome 12 (BTA12) marked by the erythrocyte antigen B locus. Rocha *et al.* (1992) localised QTLs to the region of BTA15 marked by parathyroid hormone and to the region of BTA19 marked by growth hormone 1 (*GH1*) where the genotype of the dam influenced calf weaning and birth weights, respectively.

The localisation of QTLs to chromosome intervals flanked by markers may facilitate the application of marker-assisted selection (MAS) to uncouple growth relationships at different stages of maturity and to improve carcass composition and quality characteristics. In this study, we present a positional candidate gene analysis using *GH1* as a model for QTL effects localised to BTA19 by interval analysis in a

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cross among *Bos taurus* and *Bos indicus* cattle. Our strategy involved the: (1) systematic polymerase chain reaction (PCR)-based screening of our bovine bacterial artificial chromosome (BAC) library (Cai *et al.* 1995) for clones containing *GH1*, (2) verification of the identity of a clone (BAC110R2C3) as containing *GH1* through sequence analysis, (3) physical mapping of *GH1* by FISH, (4) generation of a novel microsatellite (*KHGH1*) from BAC110R2C3, (5) integration of *KHGH1* into our framework genetic map of BTA19, (6) interval analysis of growth, carcass composition and quality characteristics against the BTA19 map to localise the position of the detected QTLs relative to *KHGH1* and *GH1*, and (7) permutation tests to ascertain the experiment-wise type I significance level associated with detected QTL effects. We propose the development of novel microsatellites for candidate genes rather than single-strand conformation polymorphisms (SSCPs) or PCR-RFLPs because of the high inherent polymorphism information content of microsatellites, which maximises their utility when typed in multiple resource families. Our results suggest that this strategy defines an efficient and cost effective approach for the positional candidate cloning of QTLs in domesticated livestock species.

Materials and methods

PCR-based BAC library screening

Nuclear sequence for bovine *GH1* was available from the Entrez database (accession no. M57764) and primers (5'-ACG CGC TGC TCA AGA AC-3' and 5'-GGC TGG AAC TAA GAA CC-3') and PCR reaction conditions (annealing temperature 58 °C; annealing time 1 min) to amplify a 441 bp fragment of *GH1* are published (Unanian *et al.* 1994). A PCR-based screening of our bovine BAC library (Cai *et al.* 1995) identified a clone (BAC110R2C3) which was verified as containing *GH1* by cycle sequencing the PCR fragment on an ABI 373 automated sequencer and aligning the obtained sequence with the published *GH1* sequence. The library currently contains 60 000 clones with an average insert size of 153 kb and a 95% probability of containing a single copy sequence and is publicly available for collaborative research.

Physical mapping of BAC clones

Chromosomal FISH procedures followed standard protocols (Pinkel *et al.* 1986) with only slight modifications and have been described

elsewhere (Yeh *et al.* 1995). Bovine chromosomes are identified according to the domestic cattle standard nomenclature (ISCNDA 1990; Popescu *et al.* 1996). Assignment of probe hybridisation signal to a chromosome band location was accomplished through examination of a minimum of 10 mitotic cells/probe and by plotting the probe signal relative to the standard ideogram.

Development of novel microsatellites from BAC clones

Methods to acquire novel microsatellites from BAC clones follow standard procedures (Cai *et al.* 1995; Marquess *et al.* 1997). A complex dinucleotide repeat microsatellite (GT)₁₅(AG)₂(GT)₂ designated *KHGH1* was isolated from BAC110R2C3 and primers were designed (5'-GGG GCC TTT GGG ATA ACC TC-3' and 5'-GGC TCC CAA CTT TGT TAT TTC C-3') to amplify a 110 bp product containing this microsatellite. Reaction conditions included denaturing at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s for 35 cycles, followed by a final elongation step at 72 °C for 10 min.

Resource family structure and phenotypes

The Texas A&M University's three generation Angleton mapping population includes 529 fullsib progeny from 39 backcross families and two F₂ families produced by embryo transfer from 80 Brahman, Angus and F₁ parents and grandparents. These families were constructed for the primary purpose of localising QTLs associated with variation in growth, carcass composition and quality traits. Carcass evaluation data are obtained at slaughter after about 140 days on feed, according to United States Department of Agriculture specifications (USDA 1989) and include maturity, marbling, quality grade, yield grade, subcutaneous fat thickness, ribeye (*l. dorsi*) muscle area, percentage kidney-pelvic-heart fat, carcass weight and dressing percentage. Tissue samples are analysed for extractable lipids, Warner-Bratzler shear force, sensory panel analysis, calcium dependent protease analysis, fatty acid and cholesterol composition of the *l. dorsi*. From the percentages of saturated (C16:0, palmitic and C18:0, stearic) and unsaturated (C18:1, oleic) fatty acids within subcutaneous fat deposits overlying the *l. dorsi* muscle at the fifth to eighth thoracic vertebrae, we derived $R1 = (100 \times C18:1)/(C18:0 + C18:1)$ and $R2 = (100 \times C18:1)/(C16:0 + C18:0)$ to represent the

percentages of long chain fatty acids that are unsaturated and of unsaturated to saturated fatty acids, respectively.

Genetic map construction

Twelve microsatellite loci, the erythrocyte antigen T and a *GH1-TaqI* RFLP which span BTA19 (Theilmann *et al.* 1989; Ma *et al.* 1996; Barendse *et al.* 1997; Kappes *et al.* 1997) were scored in the Angleton resource families according to published conditions. The locus descriptions, number of alleles, direct count heterozygosities, polymorphism information content (PIC) values and number of informative meioses for these loci are presented in Table 1. Protocols for scoring genotypes and for the construction of the genetic map of BTA19 using CRI-MAP V2.4 (Green *et al.* 1990) followed established procedures (Beever *et al.* 1996).

Interval analysis

We have developed computer software to perform interval analysis in reciprocal backcross and F₂ populations utilising the algorithm of Haley & Knott (1992). For each animal, three generation pedigree, parental breed information and computer output from the CHROMPIC option of CRI-MAP V2.4 were used to assign the breed of origin of alleles on the maternally and paternally inherited chromosomes for each marker locus in the estimated order from the sex average genetic map of BTA19. These identity-

by-descent maps and the multipoint estimates of recombination between BTA19 loci were then used to determine partial regression coefficients for each animal representing the expected contribution of breed additive (*a*) and dominance (*d*) contributions to the genotypic mean as the position of a putative QTL was moved in 1 cm increments along the chromosome. For each dependent variable, the fitted linear model included effects of gender, breed type (four levels for reciprocal backcross, and one for F₂), family nested within breed type and partial regressions on age, number of days on feed (for postslaughter phenotypes) and breed additive and dominance effects.

Permutation tests

Permutation tests as described by Churchill & Doerge (1994) with *n* = 1000 data permutations were performed for each phenotype interval analysis to determine the experiment-wise type I significance level associated with the detected QTL effects.

Within breed allelic variation at *GH1*

The interval analysis contrasts the average effect of Angus and Brahman alleles segregating in the resource families. This analysis has the greatest power for detecting a QTL when the breeds are fixed for alternate alleles, but will probably fail to detect a QTL when the breeds possess QTL alleles at similar frequencies. To test for the

Table 1. Characteristics of bovine chromosome 19 markers

Locus	No. of alleles	Heterozygosity ^a	PIC ^b	No. of informative meioses (phase known)
<i>BM6000</i> ^c	6	0.474	0.527	573 (420)
<i>HEL10</i> ^d	7	0.792	0.765	680 (494)
<i>TGLA51</i> ^d	10	0.650	0.726	788 (691)
<i>TEXAN12</i> ^e	7	0.680	0.804	323 (242)
<i>BP20</i> ^c	6	0.785	0.786	849 (727)
<i>UWCA40</i> ^d	7	0.575	0.670	724 (633)
<i>DIK39</i> ^d	8	0.662	0.738	698 (571)
<i>MAPT</i> ^c	6	0.731	0.693	603 (457)
<i>KHGH1</i>	3	0.333	0.345	283 (234)
<i>GH1</i> ^d	4	0.531	0.483	227 (0)
<i>EAT</i> ^c	2	0.471	0.295	148 (13)
<i>URB59</i> ^d	10	0.550	0.586	585 (442)
<i>DIK42</i> ^d	6	0.628	0.560	505 (316)
<i>BMC1013</i> ^c	4	0.632	0.551	562 (414)

^aDirect count heterozygosity in parents and grandparents.

^bPIC, polymorphism information content in parents and grandparents.

^cKappes *et al.* (1997).

^dBarendse *et al.* (1997).

^eBurns *et al.* (1995).

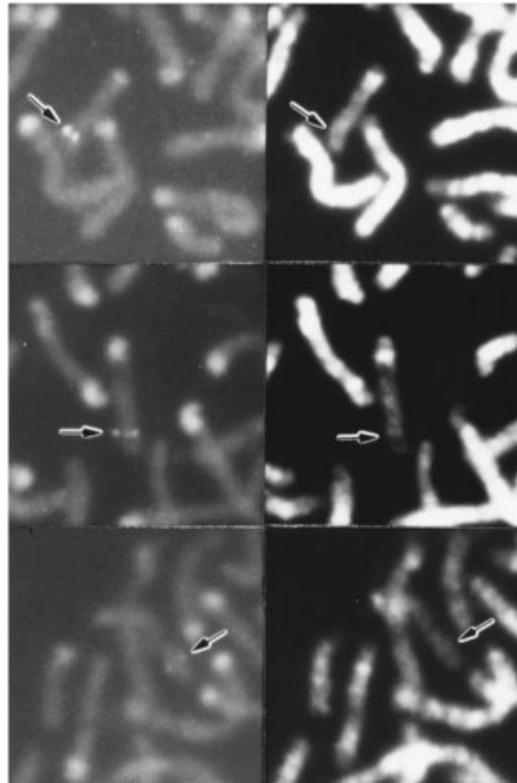


Fig. 1. Physical assignment of BAC110R2C3 to bovine chromosome 19q22 by FISH. The three partial metaphase spreads reveal probe signal and propidium iodide counterstaining (left), and associated QFH-banded chromosomes (right). Arrows indicate sites of probe hybridisation.

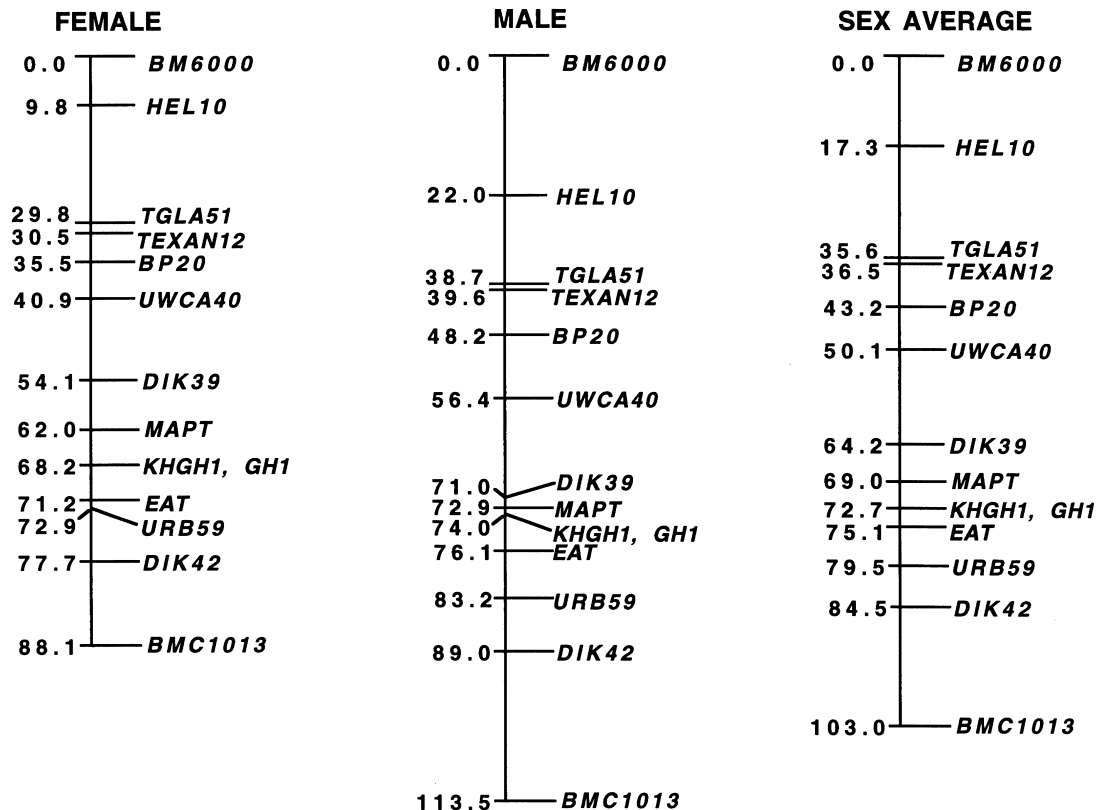


Fig. 2. Sex-average, female and male genetic maps of bovine chromosome 19.

presence of within-breed allelic effects of the *GH1* gene on growth and carcass characteristics, we employed a fullsib family based design (Soller & Genizi 1978). This design utilised the interval analysis model, but with breed additive and dominance effects replaced by effects for *GH1* genotype and for *GH1* genotype nested within family. The effect for *GH1* genotype estimates the AA, AB and BB genotypic values across families, whereas the effect for *GH1* genotype nested within family allows for differences in the genotypic values within families due to allelism within the Angus and Brahman parents. The family based analyses of growth and carcass characteristics used observations only on individuals that were completely informative for genotype at *GH1* as determined from the identity-by-descent maps for the *KHGH1* and *GH1-TaqI* markers.

Results

Physical assignment of BAC110R2C3

The *GH1* PCR product from BAC110R2C3 possessed a 100% sequence identity with the published bovine *GH1* sequence, confirming that BAC110R2C3 contained *GH1*. BAC-110R2C3 was assigned to BTA19 subchromosomal region band 22 through single colour FISH to mitotic

chromosomes (Fig. 1). This result refines the previous assignment of *GH1* to 19q17-qter (Eggen & Fries 1995) and indicates the utility of BAC clones as physical mapping reagents.

Genetic map of BTA19

Sex-average, female and male genetic maps developed from an average of 539 informative meioses on 14 BTA19 loci are presented in Fig. 2. All loci except *EAT* entered the map with support for locus order of $\text{LOD} > 3.0$. No recombinants were detected between *KHGH1* and *GH1*. The locus order in Fig. 1 agrees with the published maps (Ma *et al.* 1996; Barendse *et al.* 1997; Kappes *et al.* 1997), except *GH1* was placed proximal to *MAPT* and *EAT* by Kappes *et al.* (1997) and *MAPT* was placed distal to *EAT* by Ma *et al.* (1996). The sex-average map had an average interval of 7.9 cm and a total length of 103.0 cm which was approximately 9 cm and

22.7 cm longer than the maps of Kappes *et al.* (1997) and Ma *et al.* (1996). The map distance of 67.3 cm from *HEL10* to *DIK42* was 17.8 cm less than estimated by Barendse *et al.* (1997). The female (88.1 cm) and male (113.5 cm) map lengths differed ($\chi_{14}^2 = 49.38$; $P < 0.001$) due to increased recombination in the centromeric and telomeric regions of the male map. While not statistically different, Barendse *et al.* (1997) estimated the male BTA19 map to be 13.5 cm shorter than the female map.

Interval analysis

Figure 3 presents the interval analysis support maps for putative QTLs on BTA19 influencing growth and fatness characteristics. The estimated map position, maximum LOD score, experiment-wise significance level for the presence of a QTL, number of observations, estimated genotypic effects and within breed

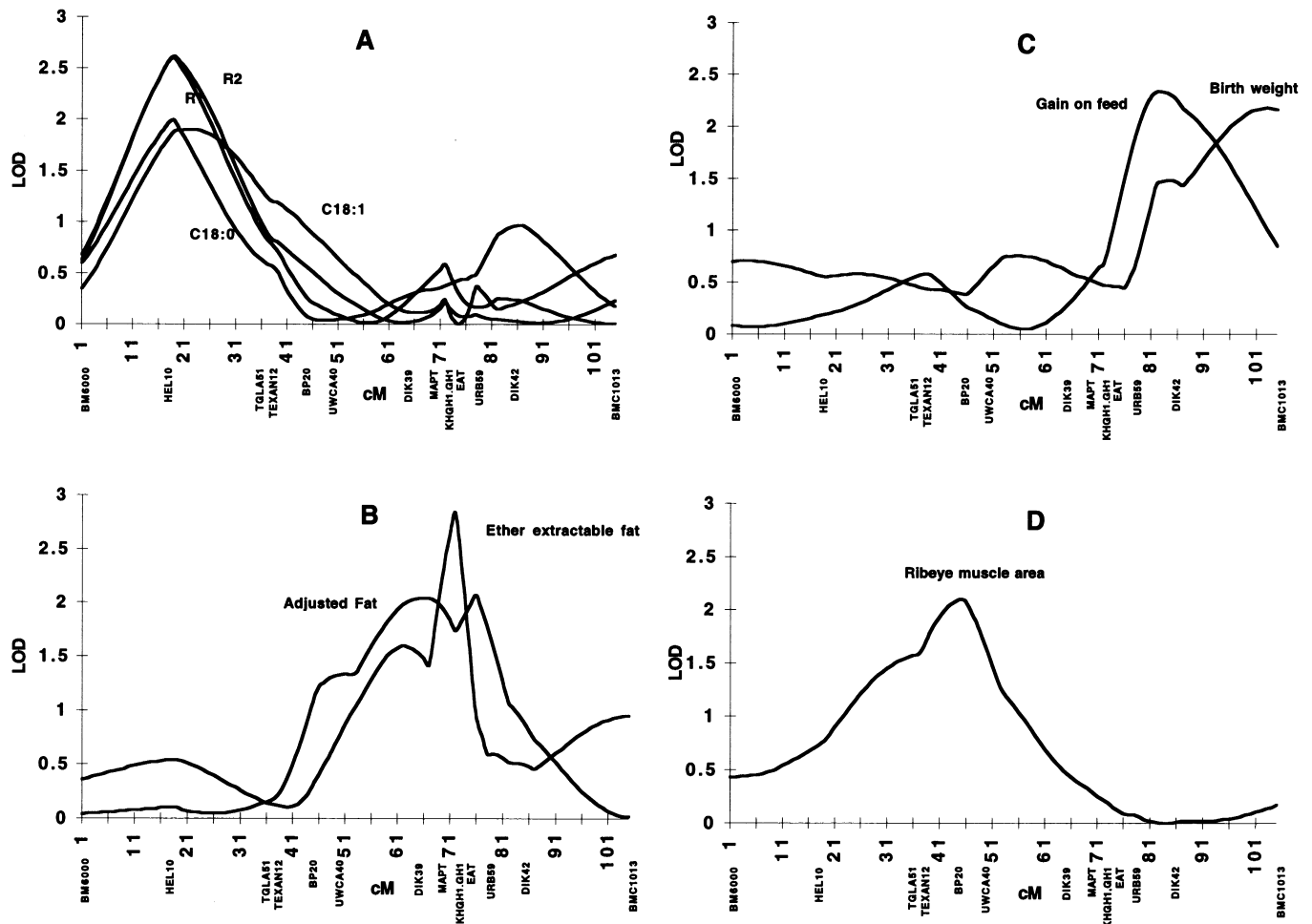


Fig. 3. Interval analysis support maps for BTA19 QTLs influencing: (A) saturated and unsaturated fatty acid composition of subcutaneous fat. *R1* is the percentage of long chain fatty acids within subcutaneous fat that are unsaturated and *R2* is the relative percentage of unsaturated to saturated fatty acids; (B) subcutaneous fat depth and ether extractable fat from the *l. dorsi* muscle; (C) birth weight and average daily weight gain in the feed lot; and (D) ribeye muscle cross-sectional area.

Table 2. Map position, maximum LOD scores, experiment-wise significance level, number of observations, QTL additive and dominance genotypic effects, and phenotypic standard deviation for BTA19 interval analyses

Trait	Map position ^a (cm)	LOD	Experiment-wise significance ^b	<i>n</i> ^c	<i>a</i> ^d ± SE	<i>d</i> ^e ± SE	σ_P ^f	$ 2a /\sigma_P$
Birth weight (kg)	102	2.18	<i>P</i> < 0.101	522	-0.30 ± 0.57	-1.74 ± 0.58	5.38	0.11
Gain on feed (kg/day)	82	2.33	<i>P</i> < 0.075	351	-0.069 ± 0.023	-0.002 ± 0.024	0.195	0.71
Adjusted fat (mm)	75	2.07	<i>P</i> < 0.023	445	-1.13 ± 0.39	-0.15 ± 0.41	3.85	0.59
Ether extractable fat (%)	71	2.84	<i>P</i> < 0.005	273	0.36 ± 0.16	-0.43 ± 0.17	1.22	0.58
Ribeye muscle area (cm ²)	44	2.10	<i>P</i> < 0.051	450	2.19 ± 0.79	0.78 ± 0.79	7.68	0.57
Stearic acid (C18:0) (%)	18	1.99	<i>P</i> < 0.180	190	-1.20 ± 0.45	0.36 ± 0.47	2.83	0.85
Oleic acid (C18:1) (%)	21	1.90	<i>P</i> < 0.030	190	1.84 ± 0.70	-0.54 ± 0.73	4.25	0.87
<i>R1</i> ^g (%)	18	2.60	<i>P</i> < 0.018	190	2.17 ± 0.73	-0.81 ± 0.75	4.54	0.95
<i>R2</i> ^h (%)	18	2.62	<i>P</i> < 0.049	189	10.54 ± 3.52	-4.24 ± 3.66	22.10	0.95

^aAt which the LOD score is maximised.

^bFrom *n* = 1000 permutations (Churchill & Doerge 1994).

^cNumber of observations.

^dQTL genotypic value of Angus homozygotes defined such that $2a = AA - BB$.

^e*AB* heterozygote deviation from QTL homozygote midpoint such that $d = AB - \frac{1}{2}(AA + BB)$.

^fWithin breed type and QTL genotype phenotypic standard deviation.

^gPercentage of long chain fatty acids that are unsaturated ($100 \times C18:1 / (C18:0 + C18:1)$).

^hPercentage of unsaturated to saturated fatty acids ($100 \times C18:1 / (C16:0 + C18:0)$).

type and QTL genotype phenotypic standard deviations for each of these QTLs are reported in Table 2.

Permutation tests

Churchill & Doerge (1994) suggested that *n* = 1000 data permutations were sufficient to ascertain the value of the test statistic corresponding to an $\alpha = 0.05$ experiment-wise type I error rate. We detected considerable variation in the value of the LOD score corresponding to an $\alpha = 0.05$ significance level for each analysed phenotype. These ranged from 1.80 for the oleic acid content to 2.68 for the stearic acid content of subcutaneous fat.

Fullsib family based analyses

Results of the family based analyses supported the results of the interval analyses for the *GH1* effects detected across families (Fig. 3). The effect of *GH1* nested within family was only significant for Warner–Bratzler shear force (*P* < 0.007), a measure of *I. dorsi* muscle tenderness.

Discussion

We detected limited support (experiment-wise *P* < 0.05) for two distinct QTLs on BTA19. For the first QTL, which was localised to the region harbouring *GH1*, *Bos indicus* vs. *Bos taurus* genotypes influence subcutaneous fat thickness adjusted for the relative amounts of fat in other

carcass depots (USDA 1989) and the percentage of ether extractable fat from the *I. dorsi* muscle (Fig. 3; Table 2). For the second QTL, *Bos indicus* vs. *Bos taurus* genotypes influence the unsaturated to saturated fatty acid profile of subcutaneous fat overlying the *I. dorsi* muscle at the fifth to eighth thoracic vertebrae (Fig. 3; Table 2). The support of *P* < 0.023 for the QTL at 75 cm from *BM6000* affecting adjusted fat thickness is modest, however, evidence for a single gene influencing both fat depots is enhanced by the detection of an effect at 71 cm from *BM6000* affecting ether extractable fat with a support of *P* < 0.005 (Table 2). Angus homozygotes were estimated to have 2.3 mm less subcutaneous fat but 0.72% greater extractable fat from the *I. dorsi* muscle than Brahman homozygotes. The importance of adjusted fat thickness lies in its role in determining the value of a carcass through its relationship to predicted yield of closely trimmed retail product. The QTL influencing adjusted fat thickness was primarily additive in effect, but that influencing extractable fat was completely dominant ($d = -a$) with heterozygotes and homozygous Brahman individuals producing the least extractable fat. Yao *et al.* (1996) found a T→C transition in the third intron and an A→C transversion in the fifth exon of the *GH1* gene and designed PCR primers to detect these alleles as single-strand conformation polymorphisms. Substitution effects for both of these alleles were found for the milk, milk fat and milk protein yields of Holstein cows (Yao *et al.* 1996). Consequently, both studies provide

evidence implicating *GH1* in the regulation of fat synthesis in cattle.

The QTL influencing the proportions of unsaturated to saturated fatty acids in subcutaneous adipose tissues ($P < 0.05$) was located 18 cm from the centromeric marker *BM6000* and the interval analysis (Fig. 3) excludes *GH1* as a potential candidate gene. The magnitude of the difference between Angus and Brahman homozygotes is substantial ($0.95 \sigma_P$ for the oleic to oleic and stearic fraction and $1.02 \sigma_P$ for the oleic to palmitic and stearic fractions). Individuals homozygous for Angus alleles at this locus possessed 2.4% less stearic acid and 3.68% more oleic acid in their subcutaneous adipose tissue than individuals homozygous for Brahman alleles (Table 2). In view of the role of dietary saturated fats in human coronary heart disease and of the fact that 55% of cattle in the Southern US possess some degree of *Bos indicus* breeding, this finding may lead to some future role in designing breeding programmes to produce meat products with an enhanced nutritive value. Acetyl-CoA carboxylase and fatty acid synthase should be tested as candidate genes for this QTL.

Considered individually, the statistical support for QTLs influencing birth weight and average daily gain on feed is very weak. However, results for both characters are presented in Fig. 3 and Table 2 since they jointly support the presence of a growth associated QTL towards the telomere of BTA19. Although the support is weak, data are also presented for a QTL putatively influencing ribeye muscle area. With the exception of ether extractable fat and adjusted fat, we conclude that there is no compelling evidence for associations between breed-specific Angus and Brahman *GH1* alleles and any of the growth, carcass composition and quality characters considered in this study. However, our findings do not exclude the possibility that QTL allelism exists within the *Bos taurus* and *Bos indicus* types that could not be detected in the analysis. However, this experiment is on-going and, with the exception of *GH1*, attempts to detect within breed allelism were not attempted.

The family-based analyses supported the across family *GH1* results reported in Fig. 3. Further, the effect of *GH1* genotype nested within family was found to be significant ($P < 0.007$) only for Warner–Bratzler shear force. In four of the six informative Angus backcross families, individuals homozygous for Angus alleles at *GH1* had lower *l. dorsi* muscle shear forces than individuals heterozygous for Angus and Brahman alleles. In five of

the nine informative Brahman backcross families, heterozygotes had lower shear forces than individuals homozygous for Brahman alleles; however, in two families the relationship was reversed and in two families there was no difference in shear force between genotypes. Within the informative F_2 family, individuals homozygous for Angus *GH1* alleles had the highest shear force, followed by heterozygotes and individuals homozygous for Brahman alleles. Since no evidence was found in the interval analysis for an across family QTL effect on Warner–Bratzler shear force, this result suggests that within-breed allelic variation exists either at *GH1* or a closely linked QTL that influences shear force.

Our approach to candidate gene analysis can either exclude a gene as being a QTL, or can provide varying degrees of support for the candidate gene–QTL relationship through proximity of the gene to the most likely position of the QTL from the interval analysis. However, the candidate gene–QTL relationship cannot be unequivocally established, even if the identified QTL effect and the map position of the candidate gene are coincident. Where the evidence supports identity, a comparative sequence analysis of the Brahman and Angus alleles present in the F_1 parents could be used to identify allelic differences potentially associated with the QTL effect. These alleles will likely differ at several nucleotide positions, and the parsimony based approach of Templeton *et al.* (1992) could be used to identify the mutational event putatively associated with the QTL effect. Parsimony reconstructs the evolutionary relationships and the hypothetical ancestral conditions among the alleles and, when the estimated additive effects of each of the alleles in the F_1 parents is superimposed on the phylogeny, groups of individuals with related alleles can be tested for a shared phenotypic effect. PCR-based tests to detect the putative allelic mutations could then be tested in independent families to refute or gain further support for the allele candidate gene–QTL relationship. If the candidate gene is not the QTL, alternate QTL–marker allele phase relationships will be detected among test populations. However, if the candidate gene is the QTL and epistasis is limited, alleles identified as favourable in our resource families should be found to be favourable in independent families. Strong linkage disequilibrium and the conservation of QTL–candidate gene allele phase relationships between populations could also lead to this result, but in either scenario, MAS could proceed using the developed test.

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