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A mammary gland EST showing linkage disequilibrium to a milk production QTL on bovine Chromosome 14

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Abstract. As part of a genome scan, ESTs derived from mammary gland tissue of a lactating cow were used as candidate genes for quantitative trait loci (QTL), affecting milk production traits. Resource families were genotyped with 247 microsatellite markers and 4 polymorphic ESTs. It was shown by linkage analysis that one of these ESTs, KIEL_E8, mapped to the centromeric region of bovine Chromosome (Chr) 14. Regression analysis revealed the presence of a QTL, with significant effect on milk production, in this chromosome region, and analysis of variance showed no significant interaction of marker genotype and family. The estimated significant differences between homozygous marker genotypes were 140 kg milk, −5.02 kg fat yield, and 2.58 kg protein yield for the first 100 days of lactation. Thus, there was strong evidence for a complete or nearly complete linkage disequilibrium between KIEL_E8 and the QTL. To identify the biological function of KIEL_E8, we extended the sequence for 869 bp by 5′-RACE. A 560-bp fragment of this shows a 90.9% similarity to a gene encoding a cysteine- and histidine-rich cytoplasmic protein in mouse. Although such a protein may have a regulatory function for lactation and a linkage disequilibrium between the EST marker and the QTL has been observed, it remains to be elucidated whether they are identical or not. Nevertheless, KIEL_E8 will be an efficient marker to perform marker-assisted selection in the Holstein-Friesian population.

Introduction

Several QTL associated with some milk production traits have been identified by genome screening with microsatellite markers (Georges et al. 1995), but it is only the milk protein genes that have been cloned and thoroughly characterized (Threadgill and Womack 1990). Thus, dissecting the molecular basis of a QTL for milk production traits remains one of the most challenging tasks in livestock genomics.

A QTL with a major effect on milk yield and composition was reported in the centromeric region of Chr 14 (Coppieters et al. 1998; Heyen et al. 1999). Because of its large effect, this QTL bears particular significance for the application of fine mapping techniques and subsequent positional cloning approach.

Although the positional cloning strategy has been successful in isolating a number of human disease genes (Collins 1995) and recently also in pigs (Milan et al. 2000), it has often been supplemented successfully with the mapping of candidate genes for the trait of interest, an approach often referred to as a positional candidate gene approach (Collins 1995). In domestic animals, where gene maps are relatively poorly developed, this approach is often combined with extrapolating the dense gene maps of human and mouse by using the knowledge of evolutionarily conserved synteny groups. An example from pigs is the genetic mapping of the white coat color phenotype to Chr 8 and subsequent identification of the *KIT* gene as the underlying mutated gene (Johansson Moller et al. 1996). For traits where substantial physiological differences exist between species, it may, however, be relevant to develop transcript maps for the species of interest.

Up to now, few EST-projects have been reported in livestock species, and only two used mammary gland tissue. Le Provost et al. (1996) reported 140 genes transcribed in goat udder, and we recently sequenced and mapped 16 ESTs derived from mammary gland tissue (Karall-Albrecht et al. 2000). Because of their differential expression in a lactating mammary gland, we have the hypothesis that these ESTs are candidate genes for milk production traits. One of these markers was genetically mapped with the International Bovine Reference Panel (IBRP) families to the centromeric region of Chr 14, and it may, therefore, be a positional candidate gene for the reported milk production QTL.

In the present study, we have tested 12 bovine mammary gland ESTs as candidate genes by integrating the polymorphic markers in a genome scan with a granddaughter design to map milk production QTL. Results of the genome scan with relevance to the EST-markers, significant estimated effects of one marker, and its further characterization are presented.

Materials and methods

Resource population and recorded traits. The resource families were established within the bovine genome mapping project of the German Cattle Breeders Association (ADR, Bonn, Germany). German AI-organizations contributed 1393 semen samples from 22 paternal half-sib families, 18 German Holsteins, three German Simmentals, and 1 Brown Swiss family. The number of sons per sire ranged from 19 to 127 and was on average 53.7. Additionally, semen from three grandsires was available. Data were obtained from the United Datasystems for Animal Production (VIT, Verden, Germany) and the Bavarian Institute of Animal Breeding (Grub, Germany) for the following milk production traits: milk, fat, and protein yield, as well as fat and protein percentage. Breeding values for the first 100 days of lactation, based on at least 25 daughter records, were used for statistical analysis.

Marker genotyping and linkage analysis. Microsatellite typing of 247 markers was performed as previously described by Thomsen et al. (2000). The microsatellite marker *ILSTS039* (http://spinal.tag.csiro.au/cgi-bin/cgdlose?ILSTS39) and twelve polymorphic ESTs, expressed in mammary gland tissue (Karall et al. 1997a, 1997b; Karall-Albrecht et al. 2000) were also used for genotyping. PCR-conditions, primer sequences, and conditions for F-SSCP-analysis were described in the references mentioned above. All genotypic data were transformed to the ADRDB database and checked for typing errors by using software that analyzed the scored genotypes for Mendelian segregation (Reinsch 1999).

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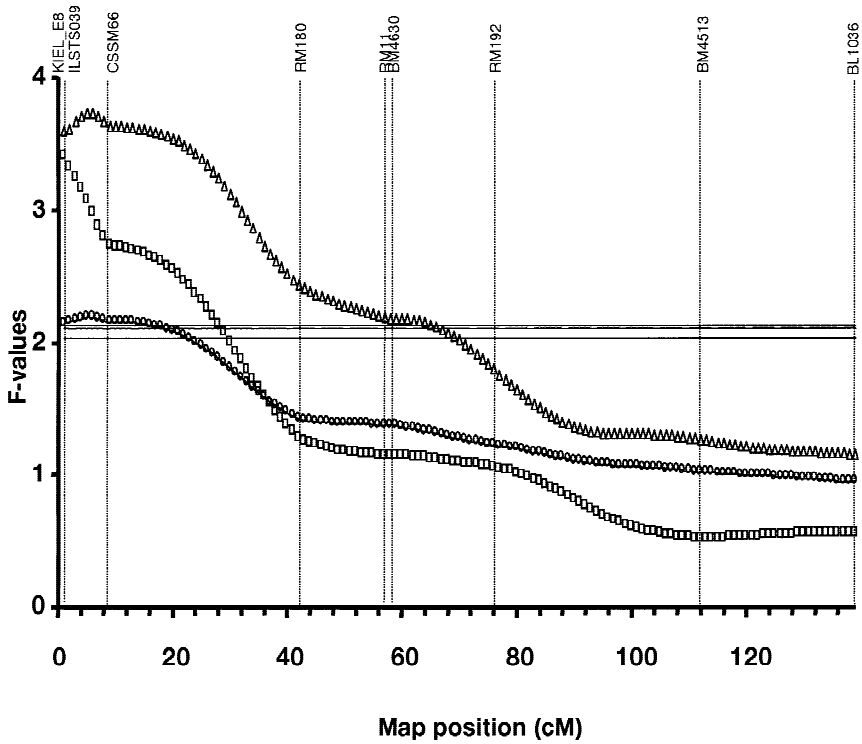

Fig. 1. F-values along the Chr 14 marker map for three different traits (△, fat yield; □, milk yield; ○, protein yield). Horizontal lines representing significant chromosomewide thresholds ($P < 0.01$) (upper line, milk yield; middle line, fat yield; lower line, protein yield).

Multipoint linkage analysis was performed by using CRI-MAP, Version 2.4 (Green et al. 1990). The FLIPS option of CRI-MAP was used to optimize the resulting gene order by altering the order of the loci and then evaluating the odds for two, three, or four loci at each time to determine the marker order with the highest log likelihood. Map distances were calculated by using the Kosambi map function as reported in the output of the BUILD option (Ott 1991).

Multimarker regression analysis. A multi-marker regression analysis based on the approach of Knott et al. (1996) was performed to analyze the effects in all families. Therefore, the following model was used:

$$Y_{ijk} = \mu + a_i + b_i X_{ijk} + e_{ijk}$$

where Y_{ijk} = estimated breeding value of bull k, son of grandsire i, marker genotype j;
μ = overall mean;
a_i = effect because of grandsire i;
b_i = regression coefficient within grandsire i
X_{ijk} = probability of the large QTL allele being transmitted from the grandsire i given the pair of detected flanking markers j of the son k; and
e_{ijk} = residual effect

The permutation approach, as suggested by Churchill and Doerge (1994), was used to determine empirically chromosomewise and experimentwise significant thresholds of the test statistic. Trait scores were shuffled among individuals within each sire family, and genotypes were retained. F-ratios were calculated at each analysis point at 1-cM intervals, and this procedure was repeated 10,000 times.

Analysis of marker-genotype effects on production traits. A two-way analysis of variance with family and marker-genotype was performed to investigate the absence and presence of interactions between both factors, as expected in the case of linkage disequilibrium or equilibrium, respectively. A model that accounted for the fixed effects of family, KIEL_E8 genotype, and the interaction between family and marker genotype was used.

The breeding values were estimated from at least 25 daughters per sire, i.e., there is little variation in the accuracy of breeding values analyzed. Differences between genotypes were estimated after eliminating the interaction term from the model. All computations were done by using the GLM-procedure of the SAS-package (SAS Institute, Cary, USA).

5′-Rapid amplification of cDNA ends (RACE) and sequence analysis. Based on the method described by Matz et al. (1999), the SMART RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, Calif., USA) was used to carry out 5′-RACE. cDNA was produced with the gene-specific primer GSP1 (5′-AGGGGAGGTGGGGCAGAGGGC-GAAGAGGCT-3′) and an adapter primer as recommended by the manufacturer with total RNA from mammary gland tissue as template. The nested gene-specific primer GSP2 (5′-GCGGGGCTGCCTACTTTT-GAATCTGGAACA-3′) and the adapter primer were used for amplification of cDNA. PCR products were cloned with the T-vector pDK101 (Kovalic et al. 1991), and inserts were sequenced with the dye terminator sequencing kit (Perkin-Elmer, Foster City, Calif., USA) and run on an ABI 377 sequencer (Perkin-Elmer). Sequences were consequently compared with EMBL/GenBank databases by using gapped BLAST (Altschul et al. 1997).

Results

Informativeness of markers and genetic mapping. Genotyping sires of the ADR-resource families with 12 ESTs revealed that the degree of polymorphism was relatively low. Four (*BTN*, *GGTB2*, *KIEL_E1*, *KIEL_E8*) of the 12 ESTs that were genetically mapped with the IBRP families showed a heterozygosity of the sires that allowed genotyping of the progenies as well. For *GGTB2* and *KIEL_E8* only two alleles, instead of three detected in the IBRP-animals, could be observed for the sires of the ADR-resource families. Consequently, the number of informative meioses for linkage mapping of the four ESTs varied from 128 to 318. Genetic mapping of the four class-I-markers is in good correspondence with the two-point linkage results of Karall-Albrecht et al. (2000). *KIEL_E1* was mapped in the interval *MILST77–BM720* on BTA13, and *GGTB2* in the interval *MCM64–BM4006* on BTA 8, and *BTN* in the interval *MB025–CYP21* on BTA23. Multipoint linkage analysis based on nine markers confirmed the location of *KIEL_E8* on Chr 14 (Fig. 1) and revealed the following order and

recombination rates between adjacent loci: *KIEL_E8*–(1%)–*ILSTS039*–(7%)–*CSSM66*–(28%)–*RM180*–(14%)–*RM11*–(1%)–*BM4630*–(17%)–*RM192*–(17%)–*BM4513*–(31%)–*BL1036*. Recombination rates were transformed into Kosambi centiMorgan as presented in Fig. 1.

Position of the QTL in the centromeric region of Chr 14. Figure 1 shows test statistics of regression analysis for milk-, fat- and protein yield on Chr 14. For all three traits the estimated F-values increase towards the centromeric region of Chr 14 and pass over the 1% chromosome-wise significance thresholds. The F-values for milk yield showed its maximum at position 0 cM, where the marker *KIEL_E8* is located, whereas F-values for protein yield and for fat yield reached their maxima at position 6 cM. In this context, it should be noted that the order of *KIEL_E8* and *ILSTS039* is uncertain, with the likelihood of the presented order only 0.34.

Association of KIEL_E8 *alleles with milk production traits.* To investigate the association between *KIEL_E8* genotypes and milk-, fat-, and protein yield, we estimated the effect within families and the overall effect. As the Brown Swiss and the three Simmental families were not informative for *KIEL_E8*, they could not be used for the estimations. For all three traits, analysis of variance revealed that there exists no family × marker genotype interaction, indicating that the *KIEL_E8* genotypes had nearly the same effects in all families and providing evidence for a linkage disequilibrium.

Absence of this interaction is documented in Fig. 2, which shows the estimated effects of the genotypes 1-2 and 2-2 in comparison with the genotype 1-1 for milk-, protein-, and fat yield within families. In families in which only the difference between two genotypes was estimated, information was contributed only from the maternal side, because sires of these families were homozygous.

The presence of the genotypes 1-2 and 2-2 is associated in almost every family with an increase of milk yield. Exceptions were observed only in the families 9, 15, and 18. Those animals with the genotype 1-2 had a lower milk yield than animals carrying the genotype 1-1, but in these three families the standard errors are 2 to 60 times higher than the estimated effects, owing to a relatively low number of animals. In all families, animals with the genotype 2-2 showed a higher milk yield.

Comparing the effects of the different genotypes on milk yield and fat yield shows that there exists an association between the inheritance of these traits within families. Although the standard errors of the estimates in families 9, 15, and 18 were relatively high, genotype 1-2 had a negative effect on both traits in these families. The presence of genotype 1-2 and 2-2 led to the reduction of fat yield, with genotype 2-2 showing a stronger effect than 1-2. Exceptions are the families 1, 3, 5, 10, and 14. In family 10, genotype 2-2 increased fat yield, whereas genotype 1-2 reduced fat yield.

The overall highly significant effect ($P < .0001$) of the marker *KIEL_E8* on the three major milk production traits is presented in Table 1. On the basis of 18 families and 1162 progeny tested bulls, a difference was estimated of 140 kg milk ($P < .0001$), −5.02 kg fat ($P < .0001$), and 2.58 kg protein ($P < .0001$) for the first hundred days of lactation between the alternative homozygous genotypes. Genotype 2-2 is associated with the highly favorable effects of increased milk and protein yield and decreased fat yield. The heterozygous genotypes show an intermediate effect on the three traits. Summarizing these results gives evidence that there exists a linkage disequilibrium between the marker *KIEL_E8* and the QTL.

Extension of the KIEL_E8 *sequence and database searches.* In order to isolate additional sequence information of the gene corresponding to the EST *KIEL_E8*, a 5′-RACE was performed that yielded a further 869 bp (AW776992) at the 5′-end. The following matches were found by using gapped BLAST (acc. no., compared sequence length, and percent of identities in brackets): *Homo sapiens* clone RP11-349C2 (AC022505, 735 bp, 91.4%); *KIAA0496* (AB007965, 106 bp, 93.4%); *Chrp* (*Mus musculus*, AJ251516, 560 bp, 90.9%); human ESTs AW602363 (242 bp, 88.9%), AI880303 (286 bp, 93.0%), AA195492 (143 bp, 95.1%); and rat ESTs AI171375 (307 bp, 89.2%), AI172358 (283 bp, 89.0%). The sequence with the database entry AJ251516 corresponds to the full-length cDNA of "a novel cysteine and histidine-rich cytoplasmic protein" (Menon et al. 2000).

Discussion

In this study the presence of the QTL affecting milk production traits in the centromeric region on Chr 14 was detected, and evidence for a linkage disequilibrium between the positional candidate *KIEL_E8* and the segregating QTL-alleles was reported.

Four of the twelve ESTs were sufficiently polymorphic to be used for genotyping the resource families and consequent QTL-mapping. That only one-third of ESTs could be integrated in the genome screen is not surprising because of the mostly diallelic nature of the markers and because of the limited genetic basis of the resource families. If these non-polymorphic ESTs are to be useful in a positional candidate gene strategy to clone QTL affecting milk production traits, it will be essential to increase the resolution of their map position. This could be done most easily by mapping them in a well-characterized whole-genome radiation hybrid panel as that described by Womack et al. (1997).

In our study, the marker *KIEL_E8* showed a significant effect on all three major milk production traits, in agreement with the effects estimated by Coppieters et al. (1998) for the QTL in the centromeric region of Chr 14. Although it is difficult to compare the different results, owing to estimating effects on the basis of similar but not totally identical traits, it is obvious that the QTL has a pleiotopic effect. Our results and those of Coppieters et al. (1998) clearly show that one QTL-allele increases milk yield and protein yield, while concomitantly reducing fat yield. Moreover, we conclude that it is quite probable that the QTL has an additive effect, because animals carrying the heterozygous genotypes are showing an intermediate performance for all three recorded traits in comparison with animals carrying the alternative homozygous genotypes.

Results concerning the position of the QTL are somewhat contradictory. Our results and those of Heyen et al. (1999) support the localization of the QTL close to the markers *ILSTS039* and *KIEL_E8*. They estimated the most likely position of the QTL affecting fat percentage in the interval *ILSTS039–CSSM66,* and a recombination rate of 2 cM between the QTL and *ILSTS039.* On the other hand, Riquet et al. (1999) applied identity-by-descent (IBD) mapping methods and reported strong evidence for the location of the QTL in the chromosome segment containing *CSSM66* and not the *ILSTS039–KIEL_E8* interval, but it should be noted that the proximal region was excluded by only one different haplotype.

As confidence intervals for locations of QTL are large and the accuracy of an IBD approach is unknown up to now, it is not surprising that there exist dicrepancies concerning the most likely positions for a QTL. Consequently, in the next phase of the project, the possibility has to be further investigated that *KIEL_E8* and the QTL are identical or are different loci in strong linkage disequilibrium. Indications supporting the first hypothesis are the expression of *KIEL_E8* in the mammary gland during lactation and the indications for a linkage disequilibrium. The absence of an interaction between the effects of family and genotype and the estimated effects within families for three traits are indications for a

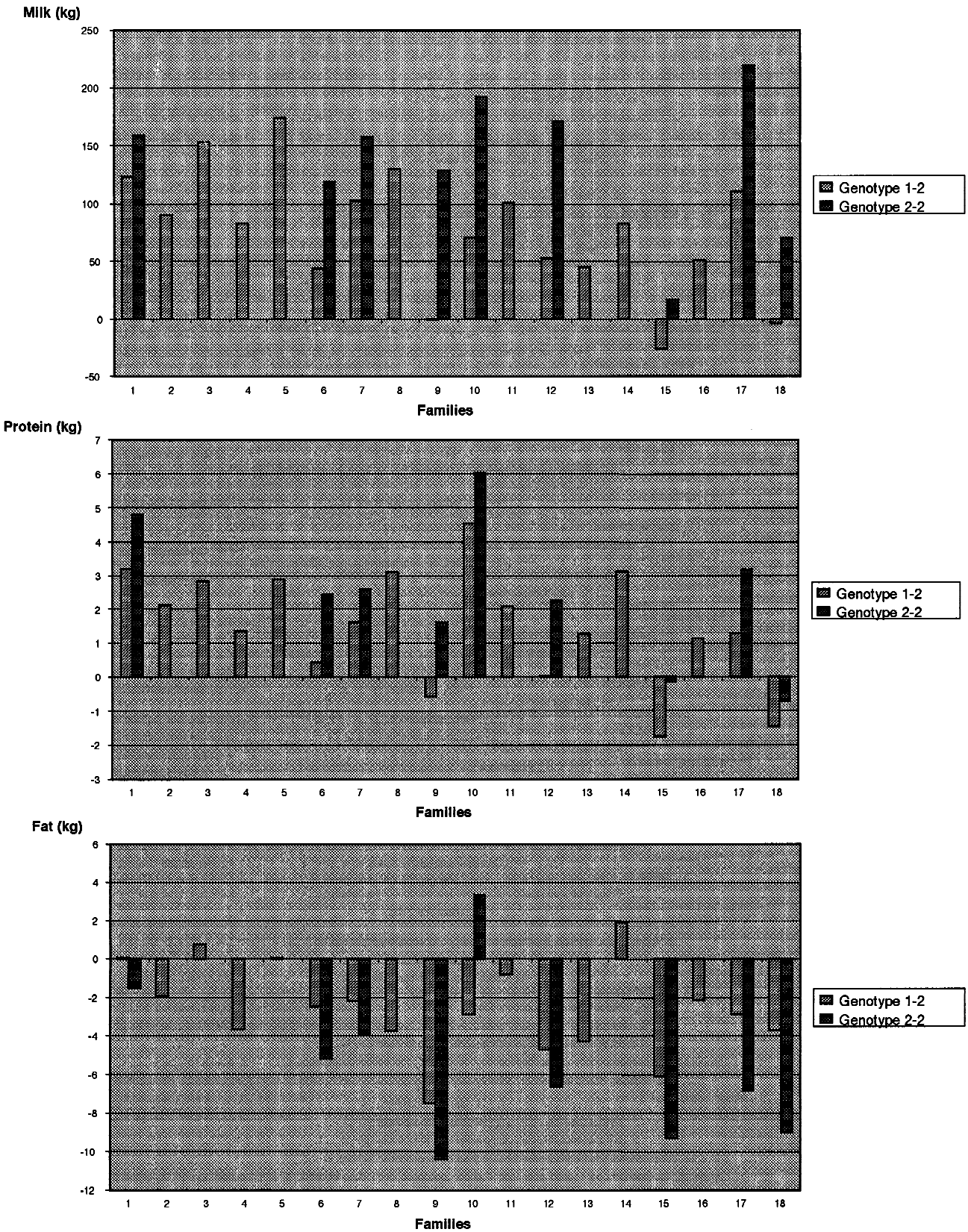

Fig. 2. Least square estimations of the effect of the *KIEL_E8* genotypes 1-2 and 2-2 in comparison with the genotype 1-1 within families for milk-, protein-, and fat-yield.

Table 1. Least square estimations and their standard errors of the effect of *KIEL_E8* genotypes on milk production traits (n = 1162).

KIEL_E8		Milk yield (kg)	Fat yield (kg)	Protein yield (kg)
Genotype 1-1	166[a]	0	0	0
Genotype 1-2	594	70 ± 9.7	−2.34 ± 0.3	1.27 ± 0.2
Genotype 2-2	402	140 ± 15.1	−5.02 ± 0.6	2.58 ± 0.3

($P < .0001$).
[a] Number of animals.

relatively strong linkage disequilibrium. In this context, it is worth mentioning that Riquet et al. (1999) reported a linkage disequilibrium extending over several tens of cM in the population analyzed by them. Thus, the second hypothesis, two loci in strong linkage disequilibrium, should also be investigated further.

In general, there are two ways to verify the alternative hypotheses: fine mapping of the QTL-region and functional analysis of the positional candidate gene. The goal of QTL fine mapping is the reduction of the confidence interval by using additional markers and more informative meioses. An alternative is the already mentioned IBD approach as performed by Riquet et al. (1999). It uses historical recombinants to identify an identity by descent (IBD) segment to define the interval harboring the QTL. As additional markers covering the target region have also to be available for this approach, it would be interesting to integrate the isolated EST *KIEL_E8* into such an approach. The alternative possibility is to identify the biological function by cross-species sequence comparison. A comparison of the original *KIEL_E8* sequence (AI461432) with the GenBank and EMBL database sequences using BLAST did not reveal sufficient similarity to known genes. We therefore extended the cDNA sequence by 5′-RACE (AW776992). DNA sequence comparison revealed similarity to a gene encoding for the protein provisionally designated as cysteine/histidine-rich protein (Chrp) (Menon et al. 2000). Chrp has a rather broad range of biological activities including DNA and RNA-binding, enzyme catalysis, protein-protein interactions, and signal transduction, because it contributes a metal-binding domain to multimeric protein. In this context, it is particularly interesting that Menon et al. (2000) speculated that galectin-3, a lactose-binding protein, may combine with Chrp to direct the lactose-galectin-3 complex into secretory pathways. Actually, in our previous study (Karall-Albrecht et al. 2000) we detected expression of a bovine homolog of galectin-1 (acc. no. AI461425).

As the function of the gene underlying the genetic variance of the three milk production traits on Chr 14 is still unknown, one can only speculate about the action and the physiological role of the expressed protein. From the fact that the QTL on Chr 14 has a major effect on three different genetically correlated traits, we assume that the QTL may have an influence on the general metabolic activity of mammary gland tissue during lactation and does not influence a specific part of a metabolic pathway.

Genetic mapping of *KIEL_E8* close to the position of the milk production QTL, the linkage disequilibrium revealed between them, and the potential function of *KIEL_E8* strengthen the gene underlying *KIEL_E8* as a positional candidate gene for the milk production QTL on Chr 14. From the fact that the detected polymorphism is located in the 3′-untranslated region, it is, however, clear that this sequence variation is not itself of biological significance. Although there is evidence for a linkage disequilibrium between the marker *KIEL_E8* and the QTL, the exact location of the marker in relation to the QTL on Chr 14 has to be further investigated. Nevertheless, from the presented results it can be concluded that the isolated EST is an excellent marker to perform marker-assisted selection in the Holstein-Friesian population.

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