A Whole Genome Scan for Quantitative Trait Loci Affecting Milk Protein Percentage in Israeli-Holstein Cattle, by Means of Selective Milk DNA Pooling in a Daughter Design, Using an Adjusted False Discovery Rate Criterion

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ABSTRACT

Selective DNA pooling was employed in a daughter design to screen all bovine autosomes for quantitative trait loci (QTL) affecting estimated breeding value for milk protein percentage (EBVP%). Milk pools prepared from high and low daughters of each of seven sires were genotyped for 138 dinucleotide microsatellites. Shadow-corrected estimates of sire allele frequencies were compared between high and low pools. An adjusted false discovery rate (FDR) method was employed to calculate experimentwise significance levels and empirical power. Significant associations with milk protein percentage were found for 61 of the markers (adjusted FDR = 0.10; estimated power, 0.68). The significant markers appear to be linked to 19–28 QTL. Mean allele substitution effects of the putative QTL averaged 0.016 (0.009–0.028) in units of the within-sire family standard deviation of EBVP% and summed to 0.460 EBVP%. Overall QTL heterozygosity was 0.40. The identified QTL appear to account for all of the variation in EBVP% in the population. Through use of selective DNA pooling, 4400 pool data points provided the statistical power of 600,000 individual data points.

LARGE half-sib or full-sib families are routinely produced in many domesticated animal species, as part of normal population reproduction procedures (dairy cattle, swine, fish) or as part of routine genetic improvement programs (poultry). These families embody great statistical power for within-population quantitative trait locus (QTL) mapping, which can be accessed by use of a "daughter design" (Soller and Genizi 1978; Weller *et al.* 1990). However, achieving the high power theoretically available with the daughter design requires genotyping very large numbers of daughters against large numbers of markers, with resultant high genotyping costs (Soller 1990) and logistical problems of sample collection.

In the case of dairy cattle, the logistical problem of sample collection has been solved in part by the use of the granddaughter design (Weller *et al.* 1990). This design is based on the availability of large half-sib families of progeny-tested sons of elite sires with readily accessible semen samples. The granddaughter design has the further advantage of reducing the sample size required for given power about fourfold as compared to a daughter design. A large number of studies employing this design have been reported (summarized in Table 7). However, the statistical power of the granddaughter design is limited by the number and size of the available sire families and is far from equaling the enormous statistical power embodied in the very large sire half-sib daughter families found in the same dairy cattle populations.

Two recent developments have opened the way to utilization of the daughter design in dairy cattle QTL mapping and its extension to other species. For dairy cattle, the high costs of sample collection from the scattered daughter families can be reduced by the use of milk samples as a source of DNA for the PCR reaction (Lipkin *et al.* 1993, 1998). Such samples are routinely collected as part of milk recording schemes and, in some instances, can be made available for QTL mapping at little additional cost.

For all species, the high costs of screening large families for marker allele frequencies can be greatly reduced by the use of selective DNA pooling (Darvasi and Soller 1994; Lipkin *et al.* 1998). In this procedure, determination of linkage between a molecular marker and a QTL is based on the distribution of parental marker alleles among pooled DNA samples of the extreme high and low phenotypic groups of the offspring population. Estimating allele frequencies in pooled DNA samples is based on a linear relation between the allele frequency in the group of individuals making up the pool and the densitometric intensity of the band corresponding to the allele. In the case of dinucleotide microsatellite markers, this requires correction for con-

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founding of main allele bands and overlapping "shadow" bands (Lipkin *et al.* 1998).

The present study follows the procedures of Lipkin *et al.* (1998) for QTL mapping by selective DNA pooling with dinucleotide microsatellites, but extends these to a complete scan of the bovine genome with respect to the target trait (milk protein percentage). In addition, a modification of the false discovery rate concept (Benjamini and Hochberg 1995; Weller *et al.* 1998) is introduced to deal with the problem of determining statistical significance in the case of multiple comparisons and to provide an empirical estimate of statistical power. The high power and low genotyping costs achieved in this study make selective DNA pooling with shadow band correction an attractive option for QTL mapping in species with large half-sib or full-sib families.

MATERIALS AND METHODS

Population and samples: The population studied was the same as described in Lipkin *et al.* (1998). Briefly, seven Israeli-Holstein artificial insemination bulls were chosen, each the sire of >1800 milk-recorded daughters. Based on estimated breeding values for milk protein percentage (EBVP%), a list of the highest and lowest 220 daughters was prepared for each sire. Milk samples of the designated daughters were obtained through the milk recording system of the Israel Cattle Breeders Association.

Two pools were prepared of the daughters at each phenotypic tail. The most extreme half of the high and low daughter groups formed the "external" high and low pools, respectively. The remaining daughters in each tail formed the "internal" high and low pools, respectively. Each pool was prepared in two independent duplicates.

Genotyping individual semen samples and individual and pooled milk samples was as described (Lipkin *et al.* 1998).

Microsatellites: A total of 138 dinucleotide microsatellite markers distributed over all 29 bovine autosomes were used in this study (web sites: U.S. Department of Agriculture, http://bos.cvm.tamu.edu/bovgbase.html; and IBRP Cattle Genome Database, http://spinal.tag.csiro.au). The distance between the markers or between the chromosome ends (centromeres and telomeres) and the closest marker averaged 17.0 cM.

Densitometric estimates of sire allele frequencies in the pools: Densitometric estimates of sire allele frequencies in the pools were obtained after correction for shadow bands, as described in Lipkin *et al.* (1998). To validate the densitometric procedure, individual milk samples in a number of the pools were genotyped. The regression and correlation of estimates of allele frequency based on pool densitometry after shadow correction and estimates of allele frequency based on individual genotyping were calculated as described (Lipkin *et al.* 1998). In addition, mean deviation of pool estimates from individual estimates was calculated as

$$[\Sigma\Sigma(I_{ij} - P_{ij})^2/n_j]^{0.5},$$

where I_{ij} and P_{ij} are, respectively, the individual or the densitometric frequency estimates of the ith allele of the jth sire-by-marker combination, and n_j is the number of alleles included in the analysis for the jth sire-by-marker combination.

Marker-QTL Linkage Tests

Comparisonwise linkage tests: *Individual sire-by-marker combinations:* Since all tests are two-tail tests, the P_{ij}, for the ith sire-by-jth-marker combination is obtained as twice the area of the normal curve from $Z(D_{ij})$ to $+\infty$, where $Z(D_{ij}) = D_{ij}/\mathrm{SE}(D_{ij})$, D_{ij} is the difference in sire-allele frequencies between the high and low daughter pools of the ith sire with respect to the jth marker (Khatib *et al.* 1994; Lipkin *et al.* 1998), and $\mathrm{SE}(D_{ij})$ is the standard error of D_{ij}, calculated as described in Lipkin *et al.* (1998). Note that this test can be applied only to sires heterozygous at the given marker.

D_{ij} values for a given sire-by-marker combination were obtained in a three-step procedure, in which the order of the steps was changed somewhat in the course of the study. At first, D_{ij} were obtained by taking the difference between the mean of the two replicates of the high external pools and the mean of the two replicates of the low external pools, as in Lipkin *et al.* (1998), and a test for marker significance carried out across the pooled Z_{ij} values for these D_{ij}. For markers having $P < 0.01$, D_{ij} and P_{ij} were also obtained for the internal pools, and finally, the results of all pools (external and internal) were combined for the definitive marker comparisonwise test. As data accumulated in the present study, however, it became clear that technical error was less than expected and that the number of daughters in the pools was the main component of $\mathrm{SE}(D_{ij})$. Consequently, the procedure was changed so that D_{ij} were initially based on one replicate each of both the external and internal pools and the second replicate analyzed only for markers having $P < 0.01$. Again, the results of all pools (external and internal) were combined for the definitive marker comparisonwise test.

Individual markers: Here, too, since all tests are two-tail tests, the P_j for an individual marker, M_j, was obtained (Weller *et al.* 1990; Lipkin *et al.* 1998) as twice the area of the chi-square distribution (d.f. = s) from χ_j^2 to $+\infty$, where χ_j^2 (d.f. = s_j) = $\Sigma Z^2(D_{ij})$ and s_j is the number of heterozygous sires tested for the jth marker. For each marker, the summation is over the subset of sire-by-marker combinations for that marker only.

Experimentwise linkage tests—the false discovery rate: For each of the above two series of tests (sire-by-marker combinations and markers), a comparisonwise error rate (CWER), or type I error P-value, was calculated for each test using standard statistical procedures, as detailed above. In the usual experimental situation, where only a small number of treatments are compared, a CWER P-value of 0.05 or 0.01 would lead to rejection of the null hypothesis (H_0 represents absence of treatment effect), with type I error likelihood $P < 0.05$ or $P < 0.01$. In the present case of linkage testing, however, multiple tests are carried out at the sire-by-marker and marker levels. Consequently, the use of a CWER based on rejecting the null hypothesis (H_0 represents no linkage) for an individual comparisonwise test at the usual CWER may result in a high proportion of false rejections among the group of rejected null hypotheses (Lander and Kruglyak 1995). That is, among the sire-by-marker combinations or markers declared to represent linkage to a QTL, a high proportion will represent false linkages. Attempting to control CWER, however, such that there is a low experimentwise probability of rejecting even one of the null hypotheses by chance alone, will result in a high proportion of false acceptances among the accepted null hypothesis. That is, a high proportion of the tests that truly represent linkage to QTL will not be recognized as such, and experimental power will be low.

As a way out of this dilemma, Benjamini and Hochberg (1995) proposed controlling the false discovery rate (FDR), *i.e.*, "the expected proportion of true null hypotheses within the class of rejected null hypotheses" (that is, the proportion of

false positive tests among the individual comparisonwise tests that are declared significant). Weller *et al.* (1998) discuss in detail the application and usefulness of this approach for QTL mapping with multiple markers. Zaykin *et al.* (2000) have pointed out that using the Benjamini and Hochberg (1995) procedure, only the unconditional FDR is controlled and not the proportion of false results among all positive tests, given that at least one test is significant. Controlling FDR at a level α based on Weller *et al.* (1998), therefore, results in controlling the FDR at a level greater than the ostensible level, α, because the proportion of false positives is expected to be greater than α if at least one test has been declared significant (Zaykin *et al.* 2000). While accepting this critique in principle, Weller (2000), however, argued that in the general class of situations where many effects are routinely declared significant, the probability of declaring at least one test significant in any specific case will be close to unity and, therefore, controlling the unconditional FDR will give similar results as controlling the conditional FDR. It is important to note that the derivation of the FDR does not require that the tests are independent (Benjamini and Hochberg 1995; Weller *et al.* 1998).

To apply the FDR approach to a particular series of comparisonwise tests, CWER *P*-values for the given comparison are ordered such that $P_{(1)} < P_{(2)} < \ldots < P_{(h)} \ldots < P_{(n)}$, where n is the total number of tests in the series (sire-by-marker or marker, as the case may be) and $P_{(h)}$ is the *P*-value corresponding to the null hypothesis of the hth test. As shown by Benjamini and Hochberg (1995), the FDR can be controlled at some level, q, by determining the largest $h = t$ for which $q < nP_{(t)}/t$. That is, under this condition, among t rejected null hypotheses, the expected proportion of falsely rejected hypotheses is no greater than q. Using this procedure, CWER *P*-values were calculated in the present study for each of the above two test series (sire-by-marker and marker). Treating each series of tests separately, the critical CWER corresponding to various desired FDR were identified. All tests having a CWER *P*-value equal to or less than the critical CWER were then taken to be significant, at the given FDR.

Estimating the proportion of false null hypotheses among all null hypotheses: For the multiple-test situations considered here, the population of all n comparisonwise tests includes two groups: G_1, comprising n_1 tests, for which the null hypothesis is false (*i.e.*, true cases of linkage), and G_2, comprising n_2 tests for which the null hypothesis is true (*i.e.*, true cases of nonlinkage). An estimate of the magnitude of n_1 and n_2 for the sire-by-marker and marker linkage test situations can be obtained on the following argument, to wit: A false null hypothesis that is wrongly accepted will nevertheless tend to have a low CWER *P*-value. Consequently, if n_1 is an appreciable fraction of n, there should be an excess of comparisons having low CWER *P*-values and a deficit of comparisons having high CWER *P*-values. The excess or deficit for any given interval, $P_h < P_j < P_k$, can be obtained as $n_{h,k} = (t_h - t_k)$, where t_h and t_k are the rank numbers of the ordered CWER test comparisons having *P*-values equal to P_h and P_k, respectively (for example: $P_h = 0.100$ and $P_k = 0.199$, Tables 2 and 7); $(t_h - t_k)$ is the number of tests having *P*-values within a defined range, and $n_2(P_h - P_k)$ is the number expected by chance for that interval out of the n_2 tests. Although n_2 is not known initially, it can be obtained by iteration. In the first iteration, set $n_2 = n$. Then, n_1 is estimated as $n_1 = \Sigma n_{h,k}$, where the summation is over all intervals for which $n_{h,k}$ is positive. In the second iteration, set $n_2 = n - n_1$ and repeat until there is no further change in the estimates of n_1 and n_2.

Adjusted FDR: When the proportion of true effects among all comparisonwise tests is large, the FDR calculated as in Benjamini and Hochberg (1995) is not appropriate. The reason for this is that the expected number of false rejections, $nP_{(h)}$, is based on the total number of comparisonwise tests, n, but, as noted above, these tests include two groups, G_1 comprising n_1 tests, for which the null hypothesis is false, and G_2 comprising n_2 tests, for which the null hypothesis is true. Thus, the expected number of falsely rejected true null hypotheses is actually $n_2P_{(h)}$, and the FDR is more appropriately calculated as $q < n_2P_{(t)}/t$, where t is the rank numbers of the ordered CWER test comparisons and n_2 is calculated as above. We use the term "adjusted FDR" for the FDR calculated in this way. Because it is conditional on some true proportion of tests for which the null hypothesis is false, we believe that the FDR calculated in this way is not subject to the critique of Zaykin *et al.* (2000) and, hence, provides unbiased estimates of the likely proportion of false positives among the tests declared to be significant.

The composition of G_1 and G_2: For sire-by-marker comparisons within markers declared to be in linkage to QTL, the composition of G_1 and G_2 is unequivocal: All comparisons for which the sire is heterozygous at the QTL belong in G_1; all comparisons for which the sire is homozygous at the QTL belong in G_2. At the marker level, however, the composition of G_1 and G_2 is more problematic. Clearly, all markers on chromosomes that do not carry even one QTL are unequivocally in G_2. Problems arise with respect to markers on the same chromosome as a QTL. Practically speaking, markers that are >35 cM from a QTL (equivalent to >25 recombination units) are relatively useless for purposes of marker-assisted selection and positional cloning, and hence one would not want to include them in G_1. For chromosomes of length 100 cM, with one randomly placed QTL, 0.42 of markers will be in this category. The proportion of markers on the chromosome that end up counted in G_1 will depend on QTL location (greater if central), and on power of the test for a marker located at the QTL. For a single centrally located QTL, having power of 0.80 at CWER 0.05 for a marker located at the QTL, about 0.65 of markers on the chromosome will end up counted in G_1. With two or three QTL on the chromosome, virtually all markers will end up in G_1. Thus, G_1 will include some markers that are in linkage to the QTL, but too far for the linkage to be useful, except as an indication that there is something of interest on the chromosome. This is part of the general problem of wide confidence intervals for QTL map location (Darvasi *et al.* 1993; Darvasi and Soller 1997) and is not specific to the adjusted FDR analysis.

Power of the test for linkage: By an extension of the argument of the previous section, it is possible to obtain an empirical estimate of power for the sire-by-marker and marker tests. Assuming that markers fall into the above G_1 and G_2 groups, then accepting the null hypothesis is a type II error for markers in G_1, but not for markers in G_2. In this case, power will equal n_m/n_1, where n_1 is as defined above, and n_m is the number of true marker-QTL linkage determinations among the group of markers having *P*-values below the critical CWER. n_m can be estimated as $n_m = t - n_2P_{(t)}$, where t, $P_{(t)}$, and n_2 are as defined above.

Estimating the proportion of heterozygosity at the QTL: Continuing this line of reasoning, the average degree of heterozygosity at the QTL was estimated by the excess proportion of significant sire-by-marker combinations among all sires heterozygous at the significant markers, as follows. At the markers showing linkage to QTL, let s be the total number of sire-by-marker combinations for which these markers were heterozygous. Then, these individual sire-by-marker combinations fall into two groups, namely: S_1, consisting of s_1 sire-by-marker combinations at which the QTL was heterozygous, and S_2 consisting of s_2 sire-by-marker combinations at which the QTL was homozygous. The ratio $H = s_1/s$ will then estimate the

TABLE 1

Regression of densitometric estimates of allele frequency in the pools, on estimates obtained by individual genotyping

BTA	Marker	Bands	Sire	No.	*a*	*b*	*r*
3	BMS963	20	1	52	−0.00	1.04	0.93
6	BMS2508	7	4	22	0.00	0.98	0.99
			6	22	0.00	0.98	0.97
			7	20	0.02	0.90	0.98
6	BM4322	9	3	23	−0.01	1.09	0.98
			6	27	−0.02	1.13	0.98
6	BM415	10	1	23	−0.01	1.09	0.97
			3	23	0.01	0.94	0.98
6	CSN3	6	3	22	−0.03	1.16	0.99
9	UWCA9	26	1	54	0.01	0.91	0.96
23	CSSM5	11	1	21	−0.01	1.06	0.92
Sire alleles only[a]					−0.02	1.00	0.93

Bands, number of bands observed on the gel and included in the shadow correction; No., number of individual genotypings; *a*, regression intercept; *b*, regression coefficient; *r*, correlation coefficient.

[a] Over all pools.

proportion of heterozygosity at the QTL. Estimates of s_1 and s_2 can be obtained by the same procedure used above to estimate n_1 and n_2, except that s_1 and s_2 are calculated for significant markers only.

The parameter H estimated in this way is an overestimate of heterozygosity for all QTL affecting EBVP% in the population as a whole. The reason for this is that H can be obtained only for significant markers. But, with only a limited number of heterozygous sires per marker, some markers in linkage to QTL will fail to reach significance, simply because none of the sires heterozygous for the marker was also heterozygous at the QTL. Taking this into account, the true proportion of heterozygosity at the QTL can be estimated as follows. Consider all markers in linkage to QTL, and let h be the true proportion of heterozygosity at the QTL to which these markers are linked and b be the average number of sires heterozygous at the linked markers. Then on the binomial distribution, the expected proportion of instances for which x of the b sires heterozygous at a marker in linkage to a QTL are also heterozygous at the linked QTL is given by $B(x) = B(x; h, b)$.

By definition, the true proportion of heterozygosity at the QTL is

$$h = \Sigma(x/b)B(x)/\Sigma B(x),$$

where the summation is from $x = 0$ to $x = b$ and $\Sigma B(x) = 1.0$. As noted above, however, for a given marker to be declared in linkage to a QTL, at least one sire heterozygous for the marker must also be heterozygous for the QTL. Thus, the observed proportion of heterozygosity at the QTL is

$$H = \Sigma(x/b)B(x)/\Sigma B(x),$$

where the summation is from $x = 1$ to $x = b$. Because $x/b = 0$ when $x = 0$, the numerator $= \Sigma(x/b)B(x)$ has the same value, whether the summation is from $x = 1$ to $x = b$ or from $x = 0$ to $x = b$. The denominator, $\Sigma B(x)$, with summation from $x = 1$ to $x = b$, however, is equal to $1 - B(0)$. Consequently,

$$H = h/[1 - B(0)].$$

Since $B(0)$ is a function only of h and b, and both H and b are known from the data, h is obtained by substituting successive values of h in the above expression, until the calculated H and observed H correspond.

The number of QTL on a chromosome: When dealing with a single F_2 or backcross population or with the progeny of a single sire, the issue of multiple QTL on the same chromosome, and thus the total number of uncovered QTL, can be resolved only by using an appropriate two-QTL interval mapping model (Zhang *et al.* 1998). Methods for application of one- or two-QTL models to interval mapping based on data obtained by selective DNA pooling are under development (Dekkers *et al.* 1999), but are not yet available. Nevertheless, as a preliminary estimate, multiple QTL were identified in this study based on additional qualitative criteria. These included: (i) the presence of one or more sires showing significance for more proximal marker(s), with clear lack of significance for more distal marker(s), while the opposite was true for other sires, and (ii) the presence of a sire showing significance for both proximal and distal marker(s), but lack of significance for intervening marker(s).

Allele substitution effects: Allele substitution effects were calculated as described (Lipkin *et al.* 1998). We were unable to derive a method to provide standard errors for these estimates.

RESULTS

Marker distribution by sires: Sire heterozygosity at the 138 markers averaged 0.67, ranging from 0.62 to 0.71. Differences in marker heterozygosity among sires were not significant (by chi-square contingency test). On the average, each sire was heterozygous at 92 markers, allowing a total of 644 individual sire-by-marker tests.

Validation of densitometric allele frequency estimates: For sixteen pools, each representing a high or low pool of a specific sire-by-marker combination, densitometric estimates of allele frequency were compared to estimates of allele frequency obtained by individual genotyping. For the most part these comparisons were for sire-by-marker combinations on BTA 6, which showed an exceptionally high number of significant effects; some comparisons were also for sire-by-marker combina-

TABLE 2

Distribution of CWER *P*-values for sire-by-marker and marker tests and derived estimates of the number of false (n_1) and true (n_2) null hypotheses among all tests

P-value	Sire-by-marker	Marker
0.000–0.099	0.234	0.442
0.100–0.199	0.104	0.109
0.200–0.299	0.106	0.094
0.300–0.399	0.090	0.109
0.400–0.499	0.070	0.036
0.500–0.599	0.073	0.051
0.600–0.699	0.079	0.022
0.700–0.799	0.067	0.043
0.800–0.899	0.076	0.036
0.900–1.000	0.101	0.058
Total no. (n)	644	138
n_1	144	81
n_2	500	57

tions on other chromosomes involving sire 1, who individually showed an exceptionally large number of highly significant effects. In each case, the densitometric estimates were based on the mean of duplicate pools. For individual genotyping, the number of genotyped daughters per pool averaged 44.8 (ranging from 18 to 78) for a total of 729 individual genotypes. Three of the sire-by-marker combinations were presented previously (Lipkin *et al.* 1998). For the remaining combinations, Table 1 shows intercepts, regression coefficients, and correlation of densitometric estimates on individual-genotyping estimates.

None of the intercepts or regression coefficients differed significantly from 0.0 or 1.0, respectively. For all alleles in the population, the correlation coefficient ranged from 0.92 to 0.99 and was 0.96 pooled over all combinations. The mean deviation of pool estimates from individual-based estimates was 0.05. For sire alleles only, the correlation was 0.93 and the mean deviation 0.06.

The proportion of false null hypotheses among all null hypotheses: Table 2 shows the distribution of CWER *P*-values for sire-by-marker and marker tests and the estimated number of false (n_1) and true (n_2) null hypotheses at the two levels of comparison. At both levels, the distribution of *P*-values differed significantly from that expected if all comparisonwise tests were generated by true null hypothesis. This indicates that the null hypothesis must be false for at least some of the tests. The estimated proportion of false null hypotheses (*i.e.*, true linkage) out of all hypotheses tested was 0.59 and 0.22, for the marker and sire-by-marker levels, respectively. These numbers indicate that more than half of all markers are in linkage to a QTL affecting trait value and that more than one-fifth of markers tested in an individual sire are heterozygous at a linked QTL affecting trait value.

TABLE 3

Critical comparisonwise error rates (CWER) according to raw and adjusted (Adj.) false discovery rate (FDR) and level of comparison: sire-by-marker and marker

	Sire-by-marker		Marker	
FDR	Raw	Adj.	Raw	Adj.
0.05	0.0033	0.0045	0.0076	0.0334
0.10	0.0087	0.0120	0.0290	0.0997
0.20	0.0258	0.0433	0.0721	0.2988

Critical CWER *P*-values according to raw and adjusted FDR: Table 3 shows critical comparisonwise *P*-values for raw and adjusted FDR of 0.05, 0.10, and 0.20, according to level of comparison. Critical *P*-values increase with increase in FDR, as expected. Critical *P*-values are greater for adjusted than for raw FDR. The difference between critical *P*-values according to raw and adjusted FDR stands in proportion to the difference between estimated number of true null hypotheses (n_2) and total number of comparisons tests (n). Thus, the difference is minor for the sire-by-marker comparisons and larger for marker comparisons.

Power of the tests for linkage: Table 4 shows total number of significant comparisons (rejected null hypotheses) and estimated power of the analysis, according to FDR (raw and adjusted) and level of comparison. In any given cell of the table, the total number of significant comparisons includes both falsely rejected null hypotheses derived from G_2 and correctly rejected null hypotheses derived from G_1. The number of correctly rejected hypotheses (*i.e.*, the number of elements of G_1 that are correctly identified as significant) is given by the number in the table, less the number of falsely rejected null hypotheses as given by the FDR. For example, at an adjusted FDR of 0.10, there are 61 rejected null hypotheses at the marker level. Of these, 6.1 represent falsely rejected null hypotheses, so that the number of

TABLE 4

Total number of significant comparisons (n_s) and power (Q) according to raw and adjusted (Adj.) FDR and level of comparison: sire-by-marker and marker

	Sire-by-marker				Marker			
	Raw		Adj.		Raw		Adj.	
FDR	n_s	Q	n_s	Q	n_s	Q	n_s	Q
0.05	42	0.28	51	0.34	34	0.41	42	0.49
0.10	59	0.38	64	0.40	41	0.49	61	0.68
0.20	84	0.49	108	0.60	51	0.58	89	0.89

TABLE 5

CWER *P*-value significance level (above) and estimated allele substitution effect, α (below), for all significant (FDR < 0.1) sire-by-marker combinations ($P < 0.012$) at the significant markers ($P < 0.100$)

BTA (cM)	Marker	Sires 1	2	3	4	5	6	7	Marker test
1 64.9	BMS4001	4×10^{-1}	4×10^{-2}	—	9×10^{-4}	—	4×10^{-1}	—	2×10^{-3}
					0.013				0.013
1 119.1	CSSM19	2×10^{-1}	1×10^{-2}	3×10^{-1}	—	1×10^{-1}	8×10^{-2}	8×10^{-1}	3×10^{-2}
			0.010						0.010
1 146.8	BMS922	1×10^{-1}	5×10^{-4}	8×10^{-1}	6×10^{-1}	—	3×10^{-1}	—	7×10^{-3}
			0.022						0.022
2 56.3	BMS1126	2×10^{-5}	—	2×10^{-2}	—	1×10^{-1}	6×10^{-1}	—	5×10^{-5}
		0.018							0.018
3 59.6	INRA3	—	3×10^{-3}	—	7×10^{-1}	—	6×10^{-1}	—	3×10^{-2}
			0.013						0.013
3 87.5	HUJII77	1×10^{-1}	—	—	—	4×10^{-1}	—	3×10^{-2}	5×10^{-2}
3 115.0	BMS896	8×10^{-3}	3×10^{-1}	—	—	—	8×10^{-1}	2×10^{-1}	5×10^{-2}
		0.011							0.011
4 24.3	RM188	8×10^{-3}	—	2×10^{-1}	5×10^{-1}	6×10^{-1}	3×10^{-1}	4×10^{-3}	5×10^{-4}
		0.012						0.013	0.013
5 55.4	RM500	1×10^{-2}	2×10^{-1}	1.00	—	2×10^{-1}	8×10^{-1}	—	8×10^{-2}
		0.009							0.009
5 76.3	BM1819	6×10^{-2}	—	4×10^{-2}	7×10^{-2}	—	3×10^{-1}	4×10^{-1}	2×10^{-2}
6 35.5	BM1329	—	—	6×10^{-2}	6×10^{-7}	2×10^{-2}	—	—	2×10^{-7}
					0.019				0.019
6 49.4	BM143	9×10^{-1}	2×10^{-8}	1×10^{-2}	3×10^{-3}	1×10^{-2}	2×10^{-1}	3×10^{-9}	2×10^{-16}
			0.022	0.014	0.013			0.028	0.018
6 76.3	BM415	4×10^{-3}	—	1×10^{-3}	3×10^{-3}	4×10^{-5}	4×10^{-2}	2×10^{-7}	3×10^{-14}
		0.012		0.016	0.012	0.022		0.025	0.017
6 82.6	CSN3	—	—	8×10^{-4}	4×10^{-4}	1×10^{-4}	6×10^{-2}	—	1×10^{-8}
				0.015	0.016	0.021			0.018
7 0.0	BM7160	—	4×10^{-3}	2×10^{-1}	—	4×10^{-1}	1.00	4×10^{-3}	2×10^{-3}
			0.013					0.014	0.013
7 59.9	UWCA20	9×10^{-3}	4×10^{-1}	2×10^{-2}	4×10^{-2}	—	5×10^{-2}	3×10^{-3}	4×10^{-5}
		0.012						0.017	0.015
7 91.1	BMS1331	3×10^{-3}	4×10^{-1}	3×10^{-2}	2×10^{-1}	5×10^{-1}	3×10^{-1}	—	8×10^{-3}
		0.013							0.013
7 116.0	ILSTS6	3×10^{-2}	—	4×10^{-6}	2×10^{-1}	1×10^{-2}	4×10^{-1}	2×10^{-1}	3×10^{-6}
				0.021					0.021
8 19.1	RM372	2×10^{-5}	1×10^{-1}	3×10^{-1}	7×10^{-1}	—	2×10^{-1}	—	3×10^{-4}
		0.018							0.018
8 38.4	BMS678	7×10^{-7}	1×10^{-1}	5×10^{-1}	5×10^{-1}	—	—	8×10^{-1}	2×10^{-5}
		0.020	0.007						0.013
8 59.0	HUJ1-74	3×10^{-2}	—	3×10^{-1}	—	6×10^{-3}	—	—	4×10^{-3}
						0.015			0.015
9 44.9	UWCA9	9×10^{2}	7×10^{-1}	—	4×10^{-1}	3×10^{-2}	—	—	8×10^{-2}
9 59.0	BMS1290	4×10^{-3}	2×10^{-1}	2×10^{-1}	4×10^{-2}	—	—	3×10^{-1}	4×10^{-3}
		0.012							0.012
9 84.6	BM4208	2×10^{-1}	—	9×10^{-1}	5×10^{-1}	—	9×10^{-1}	6×10^{-3}	9×10^{-2}
10 19.3	TGLA131	—	—	—	9×10^{-1}	4×10^{-3}	1.00	—	4×10^{-2}
						0.014			0.014
10 29.0	BRN	4×10^{-8}	5×10^{-1}	—	4×10^{-2}	6×10^{-1}	3×10^{-1}	3×10^{-1}	1×10^{-6}
		0.024							0.024
10 55.0	TGLA102	4×10^{-5}	—	—	1×10^{-2}	2×10^{-1}	5×10^{-1}	—	4×10^{-5}
		0.017			0.005				0.011
10 73.1	BMS1318	7×10^{-6}	2×10^{-3}	3×10^{-1}	—	—	9×10^{-1}	—	3×10^{-6}
		0.019	0.011						0.015
11 9.5	BM716	6×10^{-2}	4×10^{-2}	5×10^{-1}	—	3×10^{-1}	—	1.00	1×10^{-2}
11 47.7	BMS1716	—	6×10^{-1}	6×10^{-1}	8×10^{-1}	8×10^{-4}	1×10^{-1}	3×10^{-1}	2×10^{-2}
						0.017			0.017

(*continued*)

TABLE 5

(Continued)

BTA (cM)	Marker	Sires							Marker test
		1	2	3	4	5	6	7	
11 85.4	HUJVI-74	2×10^{-3} 0.019	—	8×10^{-2}	—	—	—	6×10^{-1}	5×10^{-3} 0.019
12 21.4	BMS2057	1×10^{-1}	7×10^{-1}	5×10^{-1}	1×10^{-2} 0.010	—	5×10^{-1}	5×10^{-1}	1×10^{-1} 0.010
12 49.4	BM860	3×10^{-3} 0.010	1.00	9×10^{-1}	9×10^{-1}	—	4×10^{-1}	—	9×10^{-2} 0.010
12 79.8	BM4028	4×10^{-1}	6×10^{-3} 0.013	—	—	5×10^{-1}	—	4×10^{-1}	5×10^{-2} 0.013
12 98.7	BMS1316	2×10^{-2}	3×10^{-2}	4×10^{-1}	6×10^{-1}	—	7×10^{-1}	7×10^{-1}	8×10^{-2}
13 14.8	BMS1742	1×10^{-11} 0.028	4×10^{-2}	6×10^{-1}	9×10^{-1}	—	9×10^{-1}	2×10^{-1}	2×10^{-9} 0.028
13 19.5	BMC1222	4×10^{-7} 0.022	2×10^{-1}	7×10^{-1}	—	1×10^{-1}	—	6×10^{-1}	2×10^{-5} 0.022
13 43.8	HUJ616	—	4×10^{-1}	8×10^{-1}	—	4×10^{-1}	6×10^{-1}	2×10^{-3} 0.015	4×10^{-2} 0.015
13 55.8	TGLA381	1×10^{-4} 0.016	5×10^{-1}	—	1.00	1×10^{-2}	—	—	3×10^{-4} 0.016
14 78.7	BL1036	4×10^{-5} 0.018	9×10^{-1}	7×10^{-2}	9×10^{-1}	7×10^{-1}	3×10^{-1}	7×10^{-1}	3×10^{-3} 0.018
16 11.5	HUJ614	9×10^{-2}	8×10^{-1}	2×10^{-4} 0.017	3×10^{-1}	—	—	7×10^{-2}	8×10^{-4} 0.017
16 52.8	CSSM28	3×10^{-2}	1×10^{-1}	3×10^{-2}	1.00	—	2×10^{-1}	—	2×10^{-2}
18 55.0	BMS2639	4×10^{-2}	4×10^{-1}	—	8×10^{-1}	7×10^{-1}	1×10^{-2} 0.010	1×10^{-2} 0.012	7×10^{-3} 0.011
20 31.2	BMS2461	2×10^{-1}	3×10^{-1}	—	—	—	4×10^{-2}	3×10^{-1}	9×10^{-2}
20 52.7	ILSTS72	5×10^{-2}	8×10^{-1}	2×10^{-1}	9×10^{-3} 0.10	3×10^{-1}	—	7×10^{-1}	3×10^{-2} 0.010
20 69.8	UWCA26	7×10^{-4} 0.016	3×10^{-1}	—	4×10^{-2}	9×10^{-1}	3×10^{-1}	7×10^{-1}	7×10^{-3} 0.016
21 13.0	HEL5	7×10^{-1}	4×10^{-1}	6×10^{-1}	6×10^{-1}	4×10^{-3} 0.016	3×10^{-1}	2×10^{-5} 0.021	1×10^{-4} 0.019
21 32.3	ETH131	3×10^{-4} 0.016	9×10^{-1}	3×10^{-1}	—	9×10^{-1}	—	8×10^{-1}	1×10^{-2} 0.016
21 38.6	BMS2557	—	4×10^{-2}	—	5×10^{-1}	—	—	—	9×10^{-2}
21 67.3	TGLA122	4×10^{-5} 0.017	6×10^{-2}	1×10^{-1}	6×10^{-3} 0.011	—	—	2×10^{-1}	5×10^{-6} 0.014
22 76.1	HMH1R	1×10^{-4} 0.016	7×10^{-1}	—	—	—	—	2×10^{-1}	3×10^{-3} 0.016
23 7.2	CSSM5	1×10^{-5} 0.018	4×10^{-1}	1.00	1.00	—	2×10^{-1}	—	6×10^{-4} 0.018
23 17.3	RM33	3×10^{-2}	1×10^{-1}	4×10^{-1}	—	2×10^{-1}	1.00	—	7×10^{-2}
24 33.9	ILSTS101	—	8×10^{-1}	—	—	2×10^{-2}	—	—	7×10^{-2}
26 24.8	BM1314	3×10^{-2}	—	9×10^{-1}	—	—	—	—	1×10^{-2}
26 72.6	MAF36	—	8×10^{-2}	—	2×10^{-1}	6×10^{-1}	1×10^{-2} 0.010	—	3×10^{-2} 0.010
27 0.0	BM3507	—	—	8×10^{-4} 0.015	—	—	1×10	8×10^{-1}	6×10^{-4} 0.015
27 15.0	RM209	1×10^{-1}	8×10^{-4} 0.018	—	—	9×10^{-5} 0.021	9×10^{-1}	—	8×10^{-6} 0.020
29 0.90	BMS1857	4×10^{-3} 0.013	—	—	9×10^{-1}	4×10^{-1}	3×10^{-1}	5×10^{-1}	6×10^{-2} 0.013
29 19.7	BMC8012	—	—	—	—	2×10^{-4} 0.020	—	9×10^{-1}	1×10^{-3} 0.020
29 22.8	ILSTS89	—	—	—	8×10^{-1}	—	—	2×10^{-2}	1×10^{-3}

BTA, chromosome; cM, location of the marker on the chromosome. Dashes indicate homozygous marker genotype.

truly identified marker-QTL linkages is equal to 54.9. On a raw FDR basis, power was about 0.28 to 0.49 at the sire-by-marker level and 0.41 to 0.58 at the marker level. On an adjusted FDR basis, power was 0.34 to 0.60 at the sire-by-marker level and 0.48 to 0.89 (at an adjusted FDR of 0.20) at the marker level.

Marker-QTL linkage: *Marker tests:* There were a total of 138 comparisonwise linkage tests at the individual marker level. Adjusted experimentwise FDR of 0.05 and 0.10 were obtained at a CWER of $P < 0.033$ and $P < 0.100$, respectively (Table 3). There were 42 and 61 significant markers at these levels, with estimated power of 0.49 and 0.68, respectively (Table 4).

Of the 20 additional markers included at an FDR of 0.10 as compared to an FDR of 0.05, 15 were found on chromosomes carrying at least one significant marker at FDR of 0.05. Thus, these 15 probably represent markers in linkage to QTL already identified on these chromosomes. Four more were on BTA 12, suggesting the presence of a QTL on this chromosome. These 19 markers would appear to validate the use of the less stringent criterion. The remaining one marker was found on BTA 24 that did not carry a significant marker on the more stringent criterion. We propose therefore to use the CWER providing an adjusted FDR of 0.10 as our criterion for significance in the present study.

Of the 61 significant markers on the above criterion (Table 5), 15 had two or more sire-by-marker tests significant at an adjusted FDR of 0.10; 35 had a single significant sire-by-marker test at this level; and 11 did not have even a single marker-by-sire test significant at this level.

The 61 significant markers were distributed over 23 chromosomes (Table 5): BTA 1*, 2, 3*, 4, 5*, 6*, 7*, 8*, 9*, 10*, 11*, 12*, 13*, 14, 16*, 18, 20*, 21*, 22, 23*, 26*, 27*, 29*. Of these, 18 chromosomes (indicated by an asterisk above) had at least two markers significant on the above criterion; the remainder had a single significant marker only. The large variability in significance level among sires significant for the same marker and presumably heterozygous for the same QTL (Table 5), is best explained by the nonlinear relationship of significance level to *D*-values; *D*-values and the resultant allele substitution effects show much less variation (Table 5). Nevertheless, the possibility cannot be excluded that at least in some of the instances, the significant sires are heterozygous for different alleles or for different closely linked QTL.

The proportion of significant sire-by-marker tests, for sires heterozygous at the markers, differed greatly among the sires, being 0.25 for sire 1, 0.02 for sire 6, and 0.06 to 0.10 for the remaining sires. The differences among sires were highly significant (by chi-square contingency test). A source for these differences is not apparent, as all sires were of the same breed and geographic origin and were active in the same years and same environment. Nor can the number of daughters explain these differences, as the two sires with the highest number of daughters, sire 1 (3394 daughters) and sire 6 (3407 daughters), had opposite extreme proportions of significant tests.

TABLE 6

Distribution of CWER *P*-values for sire-by-marker comparisons separately for significant and nonsignificant markers (adjusted FDR, 0.10) and derived estimates of the number of false (s_1) and true (s_2) null hypotheses among all tests

P-value	Significant markers	Nonsignificant markers
0.000–0.099	0.408	0.099
0.100–0.199	0.082	0.116
0.200–0.299	0.092	0.116
0.300–0.399	0.074	0.099
0.400–0.499	0.067	0.080
0.500–0.599	0.060	0.083
0.600–0.699	0.060	0.091
0.700–0.799	0.032	0.094
0.800–0.899	0.043	0.102
0.900–1.000	0.082	0.119
Total no. (s)	282	362
s_1	125	19
s_2	157	343

Due to the large number of significant tests obtained with sire 1, densitometric estimates of allele frequencies for this sire were confirmed by individual genotyping of four markers (BMS963, CSSM5, UWCA9, and BM415) distributed over four chromosomes and representing a wide range of *P*-values (Tables 1 and 5). The results of the individual genotypings showed a close correspondence to the densitometric estimates (Table 1).

Proportion of heterozygosity at the QTL: At the 61 markers significant at an FDR of 0.10, there were a total of 282 sire-by-marker comparisons (4.6 per marker); at the remaining 77 markers there were a total of 362 sire-by-marker comparisons (4.7 per marker). The difference in average number of sire-by-marker comparisons between significant and nonsignificant markers was not statistically significant (by chi-square contingency test). Table 6 shows the distribution of CWER *P*-values for sire-by-marker comparison tests, according to whether the marker was among the significant or nonsignificant markers. The estimated numbers of false (s_1) and true (s_2) null hypotheses at each marker group are also shown. The distribution differed significantly from expected for the significant markers, but did not differ from expected for the nonsignificant markers. The estimated number of true rejections of the null hypothesis (s_1) among the significant markers was 125; that for the nonsignificant markers was 19. Thus, the estimate of heterozygosity at QTL for the significant markers was $H = 125/282 = 0.44$. As noted, this is an overestimate,

since it includes only QTL for which at least one sire-by-marker combination was heterozygous. The corrected estimate is $h = 0.40$. Even this may be an overestimate, since it is those QTL at a lower degree of heterozygosity that would not be identified in the limited number of sires sampled from the population. The presence of more than one segregating QTL on some of the chromosomes will also contribute to inflating this estimate. In particular, chromosome 6 shows 80% significant marker-by-sire comparisons. This might be evidence of at least two QTL on this chromosome.

At an average heterozygosity of 0.40 and an average of 4.6 sire-by-marker comparisons per marker, the proportion of QTL that are not represented in heterozygous state in at least one sire-by-marker comparison is given by $(0.6)^{4.6} = 0.095$. Thus, 90% of the QTL segregating in the population will have had some opportunity to be identified in the present study.

The number of uncovered QTL: Of the chromosomes carrying significant markers, 19 (BTA 2, 3, 4, 5, 8, 9, 10, 11, 12, 13, 14, 16, 18, 20, 22, 23, 26, 27, and 29) showed only a single distinct peak of significance, consistent with the presence of a single QTL (or a single linked QTL complex) on the chromosome (data not shown). Three chromosomes, BTA 1, 6, and 21, showed qualitative indications of two separated QTL; one chromosome, BTA 7, showed qualitative indications of three separated QTL. All told, therefore, in this study, at least 23 and possibly as many as 28 QTL were found, affecting milk protein percentage in the Israeli-Holstein population.

Allele substitution effects: For each marker significant at $P < 0.100$ (FDR = 0.10, Table 3), Table 5 presents the effects obtained for all individual sire-by-marker combinations significant at $P = 0.012$ (FDR = 0.10), and the mean substitution effect of the marker, averaged over all such sires. Taking the effect of the most significant marker in any given region (when two or more adjacent markers appear to be representing the same QTL), mean effects of the putative 26 QTL averaged 0.016, ranging from 0.009 to 0.028, and summed to 0.460.

DISCUSSION

Selective DNA pooling: The results of this study confirm the effectiveness of selective DNA pooling using milk samples, for QTL mapping in dairy cattle. As found in the first stage of this study (Lipkin *et al.* 1998), pool estimates were accurate and unbiased (Table 1). As shown by Lipkin *et al.* (1998), the pools of the seven sires provided statistical power per marker equivalent to individual selective genotyping of 910 daughters per sire. Thus, the present study, involving 4396 pool genotypings, provided the equivalent of 644 (sire-by-marker tests) × 910 (daughters per sire) = 587,860 individual genotypings, a 134-fold reduction. Implications of this methodology for marker-assisted selection and fine mapping of QTL were discussed in Lipkin *et al.* (1998).

The false discovery rate approach to statistical testing: In this study a modification of the false discovery rate approach proposed by Benjamini and Hochberg (1995), which we term the "adjusted" false discovery rate, was used to deal with the problem of establishing statistical significance in a multiple test situation. It enabled decisions to be made, emphasizing control of type II rather than type I errors, without greatly increasing the proportion of false declaration of linkage in the final results. Because of the novelty of these concepts, their validity and sensitivity to assumptions has not yet been tested, and this should be taken into account when the accuracy of this methodology is assessed.

Because the FDR has as yet not been used widely for hypothesis testing, there is no consensus as to appropriate levels of FDR for various experimental situations. It would appear appropriate to take into account the relative positive contribution to the goals of the experiment of a true rejection of the null hypothesis, as compared to the negative contribution of a false rejection of the null hypothesis. In the case of QTL mapping as a preliminary for marker-assisted selection (MAS), genetic progress is proportional to the standard deviation of summed QTL value. On the simple assumption of equal effects for the different QTL, this will be proportional to the square root of the number of QTL that are followed. Thus, each additional truly identified QTL makes a positive contribution to MAS. A QTL falsely identified as present at a particular location may lead to some unnecessary genotyping costs. It will not, however, turn up as a heterozygous QTL in subsequent marker-QTL phase determination of elite sires. Hence, it will not be actively incorporated in the MAS program and will not result in lost selection intensity in other areas. Thus, for purposes of MAS, the FDR can be set relatively high. On the other hand, in the case of QTL mapping as a preliminary to high-resolution mapping or to comparative positional cloning, a false QTL identification may lead to major expenditure of wasted effort, and FDR would be set at a low level.

In the present study we chose to use an adjusted FDR of 0.10 for the marker level and 0.05 for the chromosome level. However, the general results would not have changed very much if an adjusted FDR = 0.05 had been used throughout. In contrast, the use of a Bonferroni approach would have changed results appreciably. At the marker level, an adjusted experimentwise FDR of 0.10 was obtained at a CWER of $P \leq 0.100$ (Table 3). There were 61 significant markers at these levels, with estimated power of 0.68 (Table 4). Using a Bonferroni criterion, the CWER *P*-value needed for experimentwise significance at $P = 0.10$ (assuming 50 independent marker tests) is $P \leq 0.002$. At this level, there would have been 24 significant markers (FDR = 0.005), and power would have been 0.29. Thus, although the FDR in

TABLE 7

Literature survey of significant marker-associated effects on milk production traits, in relation to effects observed in this study

BTA	Marker	Location	This study	Other studies[a]
1	TGLA49-57	1.9–46.2	—	kP (13, 20)
	POU1F1-PI	41.0	—	M, %F (29)
		46.0	—	kP (28)
	BMS4001	64.9	**	—
	CSSM19	119.1	**	—
	BMS4043	129.0	—	kP (17)
		135.0	—	M (27)
	BMS922	146.8	**	—
	BMS4014	146.8	—	M (24)
2			**	kF, M (20)
	TGLA377	27.0	—	%P (17)
	ETH121	34.4	—	%F (42), kF
	BMS1126	56.3	**	—
3			**	PrT (8)
	ILSTS096	29.7	—	M, kF, kP, %F, %P (17)
	EAL	21.6	—	M (31)
	BL41	45.2	—	%F, %P (17)
	AMY1	51.9	—	kF (3)
	TGLA263	55.6	—	%P (17, 33, 34, 42), %F (17, 33, 34), kP (41), M (16, 33, 34, 42)
	INRA3	59.6	**	—
	HUJII77	87.5	*	%P (17)
	BMS896	115.0	*	—
4	RM188	24.3	**	—
	TGLA116	48.6	—	M (12, 26)
5			**	PrT (8), kF, M (20)
	RM500	55.4	*	—
	BM1819	76.3	*	—
		97.0		M (27)
	BM315	98.6	NS	%F (17)
		131.0		kP (27)
6	VRB16	33.4	—	M (39)
	BM1329	35.5	**	%F (42)
	BM2508	44.0	—	%P, M (39)
	BM143	49.7	**	%P (36, 39, 42), M (14, 39)
	TGLA37	56.0	—	%P, %F (42), kP, kF, M (21)
	ILSTS97	67.2	—	%P (27), M (21, 27)
	RM28	74.3	—	%P (39), M (42)
	BM415	76.0	**	%P, %F (6, 7)
	CSN3	83.0	**	%P (19, 23, 38), M (19, 38) kP (15, 23, 38), %F (23, 25, 38), kF (23, 38)
	CSN1	83.0	—	%P, M (19, 24), kP, %F (24)
	AFR227	91.0	—	%P (39)
	BP7	92.0	—	%P (6)
7			**	PrT (8)
	BM7160	0.0	**	—
	BM2607	29.1	—	kF (7)
	UWCA20	59.9	**	—
	BMS1331	90.9	**	—
	ILSTS6	116.7	**	M, kP (17)
8	RM372	19.1	**	—
	BMS678	38.4	**	—
	HUJ1-74	59.0	**	
	TGLA341	75.0	—	%F (42)
	BM711	83.6	NS	%P, kP, %F (7)

(*continued*)

TABLE 7

(Continued)

BTA	Marker	Location	This study	Other studies[a]
9			**	PrT (8)
	UWCA9	44.9	*	—
	BMS1290	59.0	**	—
	TGLA427-73	57–71	—	kP, kF (13), M (42)
	BM4208	84.4	*	
10			**	Kp (20)
	TGLA111-131	0.0–19.3	—	kF (13)
	TGLA131	19.3	*	—
	BRN	29.0	**	—
	TGLA102	55.0	**	—
	BMS1318	73.1	**	—
	CSRM60	75.0	—	%P (34)
11	BM716	9.5	*	—
	BM304	24.4	—	%F, kF (7)
	BMS1716	47.7	**	—
	HUJVI-74	85.4	**	—
	LGB	108.7	—	%P (19), kP (25), %F (19, 25)
	EAJ	130.3	—	kP, kF, M (2)
12		21.0	—	M (27)
	BMS2057	21.4	*	—
		22.0	—	kP (27)
		27.0	—	kF (27)
	BM860	49.4	*	—
	BM4028	79.8	*	—
	BMS1316	98.7	*	—
13	BMS1742	14.8	**	—
	BMC1222	19.5	**	—
	HUJ616	43.8	*	
	TGLA381	55.8	**	—
14	ILSTS39	0.0	—	%P, kF, (17, 26), kP, M (26), %F (16, 17, 30)
	CSSM66	2.0	NS	%P (9), %F (9, 17, 33, 34) kF (17, 33, 34), M (9)
	TG	8.9	—	%F (9, 42), kF (42)
		56.0	—	%P (27)
	BM4305	67.4	—	%P (7), M (17)
	BL1036	79.7	**	—
	BM6425	86.7	—	M, %P, %F (6, 7)
16			**	kP, kF (20)
	HUJ614	11.5	**	—
	CSSM28	52.8	**	
		69.0	—	%F (27)
17			NS	PrT (8)
	EAF	20.7	—	%F (15)
	TGLA322	63.9	—	M (41)
18			*	M (20)
	BMS2639	55.0	**	—
	BM2078	74.4	—	kF (5)
19			**	
		48.0	—	%F (27)
	bGH	65.7	—	%P (22, 40), kP (11), kF (11, 18), M (11)
20			**	PrT (8)
		2.0	—	M (27)
	TGLA126	31.2	—	%P (13, 42), %F (42)
	BMS2461	31.2	*	—
	GHR	52.6	—	%P (11), M (1)
	ILSTS72	52.7	**	—
	UWCA26	69.8	**	%P (4)
	BMS521	75.0	—	kP, kF, M (24)

(*continued*)

TABLE 7

(Continued)

BTA	Marker	Location	This study	Other studies[a]
21	HEL5	13.0	**	—
	BM3413	15.0	—	kF, M, kP, kF (5)
	BM103	30.5	—	%P, %F (4)
	EAS	32.0	—	kF (31)
	ETH131	32.3	**	kP, M (32)
	BMS2557	38.6	*	—
	ILSTS103	43.1	—	M, kP (17)
	TGLA122	67.3	**	—
22	HMH1R	76.1	**	—
23	CSSM5	7.2	**	—
	RM33	17.3	*	—
		21.0	—	%P (27)
	BOLA-DRB3	30.0	—	%P (35), kP (35, 37), M (35)
	513	31.0	—	kF (5)
	EAM	35.4	—	%P (24), kP, M (31), %F (3)
	CYP21	36.0	NS	%F (7)
	PRL	43.2	—	%P, kP, M (10)
24	ILSTS101	33.9	*	—
25			**	
		44.0	—	kP (27)
26	BM1314	24.8	*	—
	BM4505	39.7	NS	%F, kF (5)
	MAF36	72.6	**	—
27	BM3507	0.0	**	—
	RM209	15.0	**	—
	BM203	64.1	—	%P, kP (5)
29	BMS1857	0.9	*	—
	BMC8012	19.7	**	—
	ILSTS89	22.8	*	
	ARO26	37.0	—	kP, M (17)
	BMC3224	43.6	—	%P (6)

kP, kilograms protein per year; kF, kilograms fat per year; %P, milk protein percentage; %F, milk percentage; M, kilograms milk per year; PrT, production traits; —, not tested; NS, not significant; *, $P < 0.10$ (FDR = 0.10); **, $P < 0.033$ (FDR = 0.05).

[a] References: (1) Aggrey *et al.* (1998), (2) Andersson-Eklund *et al.* (1990), (3) Andersson-Eklund and Rendel (1993), (4) Arranz *et al.* (1998), (5) Ashwell *et al.* (1997), (6) Ashwell *et al.* (1998a), (7) Ashwell *et al.* (1998b), (8) Boichard (1999), (9) Coppieters *et al.* (1998), (10) Cowan *et al.* (1990), (11) Falaki *et al.* (1997), (12) Georges *et al.* (1993), (13) Georges *et al.* (1995), (14) Gomez-Raya *et al.* (1998), (15) Gonyon *et al.* (1987), (16) Heyen *et al.* (1998), (17) Heyen *et al.* (1999), (18) Hoj *et al.* (1993), (19) Ikonen *et al.* (1999), (20) Kalm *et al.* (1998), (21) Kuhn *et al.* (1996), (22) Lagziel *et al.* (1996), (23) Leone *et al.* (1998), (24) Maki-Tanila *et al.* (1998), (25) McLean *et al.* (1984), (26) Medjugorac *et al.* (1996), (27) Moisio *et al.* (2000), (28) Nadesalingam *et al.* (1998), (29) Renaville *et al.* (1997), (30) Riquet *et al.* (1999), (31) Rocha *et al.* (1998a,b), (32) Ron *et al.* (1994), (33) Ron *et al.* (1996), (34) Ron *et al.* (1998), (35) Sharif *et al.* (1998), (36) Spelman *et al.* (1996), (37) Starkenburg *et al.* (1997), (38) Velmala *et al.* (1995), (39) Velmala *et al.* (1999), (40) Vukasinovic *et al.* (1999), (41) Weller *et al.* (1995), (42) Zhang *et al.* (1998).

this case would have been negligible, many true effects, snared at a somewhat higher FDR, would have been missed.

The power of the experiment and the adjusted false discovery rate: The adjusted FDR approach conceives of the population of multiple tests as consisting of two subgroups, one, which we have denoted G_1, for which the null hypothesis is false, and a second, denoted G_2, for which the null hypothesis is true. The characteristic feature of the first population is that it will tend to have lower CWER *P*-values than the second. On this basis we proposed a methodology for estimating the size of the two subgroups based on a comparison of expected and observed numbers in each *P*-value class. The excess numbers in the low *P*-value classes were assigned to G_1, the remainder to G_2. Using this approach, the estimated proportion of false null hypotheses (*i.e.*, true linkage) out of all hypotheses tested was 0.59 and 0.22, for marker and the sire-by-marker levels, respectively (Table 2). These proportions are internally consistent. If 23 of 29 (four-fifths) chromosomes carry a QTL, it is plausible that only about 50% of markers are in linkage to QTL:

20% of markers will be located on chromosomes that do not carry a QTL and, on chromosomes carrying a single QTL, some markers will be too far from the nearest QTL to show an effect. Similarly, if about 50% of markers are in linkage, it is plausible that only about 20–25% of all sire-by-marker comparisons represent heterozygous QTL: Half of the markers are not in linkage, and of those in linkage, at least half of the sires are not heterozygous at the QTL.

By estimating the size of the two populations, it was possible to calculate an adjusted FDR, which further increased the power of the analyses. Further exploitation of this approach enabled estimates of the empirical power of the experiment to be obtained. Power at FDR = 0.10 was equal to 0.68 and 0.40 at the marker and sire-by-marker levels, respectively (Table 4). The question of power is particularly important at the sire-by-marker level, since correctly identifying sires heterozygous at the QTL is an essential component of phase determination for MAS. The rather low power at the marker and sire-by-marker levels is probably due, at least in part, to the fact that many of the markers were at a distance from the QTL to which they were linked. As a result, power for these markers (and for the sire-by-marker combinations involving them), would necessarily be low. Thus, we anticipate that power will be somewhat greater, once mapping proceeds to identify markers in tight linkage to the QTL. In addition, the high and low selected groups in this experiment included only the high and low 10% of the daughter population. Including 15% rather than 10% adds somewhat to mapping power (Darvasi and Soller 1992). Thus, a combination of more tightly linked markers and expanded high and low groups may be able to increase power of the pool analyses at the sire-by-marker level. A combination of pool genotyping and individual genotyping may also provide further increase in power at this level.

Finally, through this approach we were able to estimate the average degree of heterozygosity at the QTL. This is of interest with respect to the potential impact of MAS, which will be greatest for positive alleles that are at low frequency. An average heterozygosity of 0.40 or less at the QTL, as found in this study, implies that for most QTL, allele frequency is unequal, with at least one allele at high frequency (>0.7) and the other at correspondingly low frequency (<0.3). This accords with the prior history of the Israeli-Holstein cattle population. This population was under intensive selection for high milk yield for almost 50 years. Because of the negative genetic and phenotypic correlation between milk yield and milk protein percentage, this will have resulted in strong selection against alleles with positive effects on milk protein percentage. Indeed, such selection is apparent in the higher proportion of target daughters accessed in the high EBVP% tail of the population than in the low tail (Lipkin *et al.* 1998). Thus, we can make the further inference that usually it is alleles with positive effects on milk protein percentage that are at low frequency in this population. The average proportion of heterozygosity (0.40) is quite favorable for MAS, since it implies that in each generation a significant proportion of QTL will be correctly phased at the level of the full-service sire. This will allow marker-QTL phase information to accumulate rapidly in the population as a whole.

Comparison to other studies in the literature: Because of the large scope and high power of the present study, it is of interest to compare the results of this study to those of other QTL mapping studies of milk production traits in dairy cattle (Table 7). Cross-study comparisons are complicated by the fact that the different studies used different designs and levels of significance and examined different traits. With respect to levels of significance, we will accept a QTL as "confirmed" in a given region identified by two or more independent studies. With respect to the traits involved, because of the physiological correlation between milk quantity and milk composition and the correlation among the various milk components, a single QTL can be expected to have effects on more than one milk production trait. On this basis, we will consider a marker-linked effect on any of the milk production traits (kilograms milk, kilograms protein, kilograms fat, protein percentage, or fat percentage) as indicating a QTL affecting "milk production." Comparing across studies, therefore, we propose that when the same or closely linked markers show effects on different milk production traits in different studies, the markers nevertheless be considered as reflecting the same underlying QTL. This is clearly a simplification, and, in practice, different QTL and possibly different alleles at the same QTL may differ in their effects on the various production traits. Indeed, it is the QTL alleles that break the overall correlation pattern among traits that are of greatest interest for purposes of marker-assisted selection. It is also possible that milk production QTL are clustered, so that different studies are identifying different QTL located in the same general chromosomal region, but affecting different aspects of milk production.

We review the chromosomal level, without distinction as to marker location within the chromosome. On this basis, multiple studies, reviewed in Table 7 and in Boichard (1999), have reported QTL on 20 chromosomes: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 16, 17, 18, 19, 20, 21, 23, and 29. The present study found significant evidence for linkage on all but BTA 17 and 19. On BTA 19 the growth hormone gene was linked previously to milk production traits (Hoj *et al.* 1993; Lagziel *et al.* 1996; Falaki *et al.* 1997). Although none of the markers on BTA 19 was significant in the present study, we note that sire 1 had a sire-by-marker CWER of $P = 0.03$ at MAP2C (FDR of 0.17), which is adjacent to bGH. On the other hand, the effect found by Lagziel *et al.* (1996)

was associated with a haplotype that is very rare in the Israeli-Holstein population, and it was probably missing among the seven sires in the present study.

In addition, linkage to QTL was found in the present study for three chromosomes, BTA 12, 26, and 27, for which there is only one report in the literature. Linkage was also found on two chromosomes, BTA 13 and 22, for which results have not been reported in the literature.

In summary, the present study and the literature agree in finding QTL on 21 chromosomes and in not finding QTL on three chromosomes: BTA 15, 24, and 28. There is disagreement with respect to two chromosomes for which QTL were identified in the present study, but not confirmed in the literature, and with respect to two chromosomes for which QTL were confirmed in the literature, but were not found in the present study. We believe that any discrepancy between the results of the present study and those in the literature can best be explained by the strong three-way "chance" element at play in any particular study, namely, whether a QTL segregating in the population as a whole is segregating in the sampled set of sires, whether the sampled segregating QTL is close to a genotyped segregating marker, and whether the segregating QTL/marker pair passes the significance threshold.

The proportion of total genetic variance explained by the uncovered QTL: The present study may have uncovered as many as 90% of the QTL affecting milk protein percentage that are segregating in the Israeli-Holstein population. It is of interest, therefore, to calculate the actual fraction of the total genetic variance that is explained by the uncovered QTL. The fraction of genetic variance for protein percentage that is accounted for by the QTL identified in this study will stand in proportion to $\Sigma(2p_iq_i\alpha_i^2)$, where $2p_iq_i$ is the proportion of heterozygotes at the ith QTL and α_i is the allele substitution effect. In applying this expression to the present data set, we took α_i as obtained separately for each QTL from the estimates of allele substitution effects, but $2p_iq_i$ as the average degree of heterozygosity, h, for the QTL identified in this study, so that the expression becomes $h\Sigma\alpha_i^2$.

The allele substitution effects derived in this study are in terms of estimated breeding values (EBVs). Because EBVs are regressed toward the mean (depending on the accuracy), allele substitution effects derived from EBVs will underestimate the true substitution effects (Israel and Weller 1998).

Currently there is no accepted procedure to translate allele substitution effects in units of EBVP% to the actual allele substitution effects in terms of trait value for milk protein percentage. Therefore, we propose to obtain an estimate of the fraction of genetic variance in milk protein percentage in the study population that is accounted for by the effects of the identified QTL, by keeping the calculations in terms of the variance of EBVP% (equal to the genetic variance times accuracy squared). To do this we will take allele substitution effects in terms of EBVP%, estimate variance of EBVP%, and calculate the proportion of variance in EBVP% that is explained by the observed allele substitution effects. It seems plausible that whatever the eventual translation from EBVP% to trait value for protein percentage, the relative proportion of explained genetic variance would remain the same.

An estimate of the variance of EBVP% in the study population was obtained as follows: The average difference between the high and low daughters of the various sires was 0.195 EBVP%. On the average, the high and low daughters represented the high and low 0.085 of each tail, so that the difference between the means of the two tails is about 3.6 phenotypic standard deviations. Thus, the standard deviation of EBVP% can be estimated as 0.054 (= 0.195/3.6), and the variance as 0.00292 (= 0.054^2). On a within-sire basis, only three-fourths of the genetic variation is present. Thus, within-sire variance of EBVP% represents only 0.75 of the total variance of EBVP%, giving an estimate of 0.00389 (= 0.00292/0.75) for the population variance of EBVP%, assuming between-sire variance in EBVP% is 25% of total variance in EBVP%.

Average heterozygosity at the QTL was estimated above as $h = 0.40$, and the summed squared allele substitution effects came to 0.0080, taking the allele substitution effect associated with the most significant marker in each region. Thus, the estimate of EBVP% variance is 0.00320 (= 0.0080×0.4). Since average marker spacing was about 17 cM, assuming random distribution of QTL and markers, the average QTL would be about 4 cM from the nearest marker, so that α-values are reduced by 8%. The corrected estimate of the variance in EBVP% contributed by these loci is thus 0.00373 [= $(1.08)^2(0.00320)$]. This is more or less equal to the estimated variance of EBVP% in the population (0.00389) and does not include the 10% of QTL that may have been homozygous in all sires tested and, hence, not uncovered. On the other hand, allele substitution effects of significant markers tend to have positive estimation errors and hence may be somewhat overestimated, and significant markers may be a bit more closely linked to QTL than the average marker (since more closely linked QTL have a greater chance to be detected). Because of the relatively high power of the present study, however, we believe that these effects will not be great. Assuming that the latter two effects are equal to the missing 10% of QTL, the QTL uncovered and implied in this study appear to account for the total genetic variance in EBVP% and, by inference, for total genetic variance in protein percentage in the Israeli-Holstein population.

Marker-assisted selection: The summed total of effects of the putative QTL uncovered in the present study is equal to 0.460 EBVP%. Because EBVP% are moderately regressed, the actual substitution effects at the

individual QTL may be considerably larger than their estimates (Israel and Weller 1998; Lagziel *et al.* 1999). Thus, 0.460 EBVP% represents a minimum estimate of the total summed allele substitution effects on milk protein percentage for QTL segregating in the Israeli-Holstein population. As noted above, the results of this study and the history of the Israeli-Holstein breed suggest that the average frequencies at the QTL of alleles having a positive effect on milk protein percentage are low. In this case, the summed allele substitution effects on milk protein percentage over all segregating QTL represents the lower limit for genetic increase in milk protein percentage in the population. Thus, existing genetic variation for milk protein percentage in the Israeli-Holstein population should allow an absolute genetic increase of as much as 1% in milk protein percentage. This would represent an increase of one-third above the present mean of 3%. Such an increase would, of course, be accompanied by correlated effects on yearly milk production and other traits, according to the specific pleiotropic effects of the milk protein percentage QTL.

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