

Linkage Disequilibrium Between Microsatellite Markers Extends Beyond 1 cM on Chromosome 20 in Finns

Karen L. Mohlke,^{1,6} Ethan M. Lange,^{2,6,7} Timo T. Valle,³ Soumitra Ghosh,^{1,8} Victoria L. Magnuson,^{1,9} Kaisa Silander,¹ Richard M. Watanabe,^{2,10} Peter S. Chines,¹ Richard N. Bergman,⁴ Jaakko Tuomilehto,^{3,5} Francis S. Collins,¹ and Michael Boehnke^{2,11}

¹Genetics and Molecular Biology Branch, National Human Genome Research Institute, Bethesda, Maryland 20892, USA;

²University of Michigan, School of Public Health, Department of Biostatistics, Ann Arbor, Michigan 48109, USA;

³National Public Health Institute, Department of Epidemiology and Health Promotion, Diabetes and Genetic Epidemiology Unit, Helsinki, Finland; ⁴Department of Physiology and Biophysics, Keck School of Medicine, University of Southern California, Los Angeles, California 90033, USA; ⁵Department of Public Health, University of Helsinki, Helsinki, Finland

Linkage disequilibrium (LD) is a proven tool for evaluating population structure and localizing genes for monogenic disorders. LD-based methods may also help localize genes for complex traits. We evaluated marker–marker LD using 43 microsatellite markers spanning chromosome 20 with an average density of 2.3 cM. We studied 837 individuals affected with type 2 diabetes and 386 mostly unaffected spouse controls. A test of homogeneity between the affected individuals and their spouses showed no difference, allowing the 1223 individuals to be analyzed together. Significant ($P < 0.01$) LD was observed using a likelihood ratio test in all (II/II) marker pairs within 1 cM, 78% (25/32) of pairs 1–3 cM apart, and 39% (7/18) of pairs 3–4 cM apart, but for only 12 of 842 pairs more than 4 cM apart. We used the human genome project working draft sequence to estimate kilobase (kb) intermarker distances, and observed highly significant LD ($P < 10^{-10}$) for all six marker pairs up to 350 kb apart, although the correlation of LD with cM is slightly better than the correlation with megabases. These data suggest that microsatellites present at 1-cM density are sufficient to observe marker–marker LD in the Finnish population.

Linkage disequilibrium (LD), the nonrandom association between alleles of linked markers, reflects the size of chromosomal segments remaining intact in a population. LD analysis has proved powerful for high-resolution mapping of disease genes for monogenic disorders, including cystic fibrosis (Rommens et al. 1989) and diastrophic dysplasia (Hastbacka et al. 1994). In the Finnish population, LD has narrowed the candidate gene interval for many recessive disorders that have one or a few predisposing alleles at a single locus (Peltonen et al. 1999).

In principle, LD also may aid in positional cloning of genes for complex traits in founder populations, particularly if one or a small number of founder alleles contributes substantially to disease risk, and if the density of markers is suf-

ficiently high. Marker–marker LD may provide an upper bound for the needed marker density by demonstrating the presence of chromosomal regions inherited together since the population was founded or subjected to a bottleneck.

The limited available experimental data report varying evidence of LD over cM distances. In one study of 50 Finns, up to one half of the marker pairs within 2 cM showed LD at $P < 0.05$ (Peterson et al. 1995). However, a study of the X chromosome in 80 Finnish males reported that only 2 of 16 marker pairs up to 2 cM apart showed significant LD ($P < 0.05$) (Laan and Paabo 1997). On chromosomes 5, 6, and 8, in an average of 986 haplotypes from Irish schizophrenia pedigrees, significant LD ($P < 0.05$) was observed in 81% of marker pairs within 1 cM and 35% of pairs 1 to 2 cM apart (Kendler et al. 1999). A genome-wide survey of LD performed with 5048 microsatellites in 54 independent chromosomes from European, Utah, and Amish CEPH families detected significant ($P < 0.01$) LD in ~4% of markers within 4 cM of one another (Huttley et al. 1999). Recently, Eaves et al. assessed LD in a 6.5-cM region of chromosome 18q21 in samples of 800 chromosomes each from Finland, Sardinia, the United States, and the United Kingdom (Eaves et al. 2000). They found LD extending up to 1 cM, with somewhat greater LD in the Finns and Sardinians. Additional data are needed in various populations and genomic regions to estimate the extent and variability of LD and to assess whether the presence of LD will be useful for fine-mapping complex disease genes.

⁶These authors contributed equally to this work.

Present addresses: ⁷Department of Public Health Sciences, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1063, USA; ⁸The Max McGee National Research Center for Juvenile Diabetes, Children's Hospital of Wisconsin, and Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI 53226, USA; ⁹Science and Technology Division, Corning Incorporated, Corning, NY 14831, USA; ¹⁰Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA.

¹¹Corresponding author.

E-MAIL boehnke@umich.edu; FAX (734) 763-2215.

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Type 2 diabetes is a common disorder that causes considerable morbidity and mortality throughout the world. Evidence for a genetic component in type 2 diabetes has been obtained using twin and family history studies (Newman et al. 1987; Rich 1990; Kaprio et al. 1992). Several groups have identified linkage signals for type 2 diabetes affection status (Ehm et al. 2000), and one underlying gene has been identified (Horikawa et al. 2000). The Finland–United States Investigation of NIDDM Genetics (FUSION) study aims to identify susceptibility genes for type 2 diabetes and related quantitative traits in the Finnish population. In this study, we ascertained type 2 diabetes-affected sibling pairs and additional relatives (Valle et al. 1998). A 10-cM genome scan on >2000 individuals revealed several regions of suggested affected sib-pair linkage (Ghosh et al. 2000), including portions of both arms of chromosome 20 (Ghosh et al. 1999). To better assess the importance of these regions, we genotyped a total of 43 markers at 2.3 cM average density on this chromosome. These data provide the opportunity to assess the degree of marker–marker LD and the distances over which LD extends. We present here microsatellite marker-to-marker LD across chromosome 20 in 1223 individuals. We show that significant inter-marker LD is virtually always observed for marker pairs up to 1 cM apart, and is generally absent for marker pairs at distances greater than 4 cM apart.

RESULTS

Forty-three microsatellite markers spanning chromosome 20 were genotyped on 837 unrelated Finns affected with type 2 diabetes and 386 of their spouses. Estimated genetic positions of the markers are shown in Table 1. The 43 markers have an average estimated heterozygosity of 0.77 and average spacing of 2.3 cM across the 99.2-cM chromosome. Table 1 also shows the number of alleles we observed for each marker and the number of alleles we analyzed when low-frequency alleles were pooled.

To determine if the affected individuals and their spouses showed different haplotype frequencies, a permutation test of heterogeneity was performed for all 903 possible pairs of the 43 markers. Among these pairs, 12 were significant at the 0.01 level, consistent with randomness. Further, the distribution of the 903 *P*-values appeared to be uniform across the interval zero to 1 (data not shown). These results suggested no important differences in haplotype frequencies exist between the affected individuals and controls, allowing us to pool data across the two samples.

After pooling the diabetic individuals and their spouses to generate a sample of 1223 individuals, we analyzed all 903 possible marker pairs using a likelihood ratio test for LD. We plotted the $-\log_{10}$ *P*-values of all 903 comparisons in the combined sample as a function of estimated intermarker distance (Fig. 1a). A total of 55 marker pairs are significant at the 0.01 level ($-\log_{10}$ *P*-value of 2), compared to nine significant results expected by chance in the absence of LD. LD between marker pairs less than 10 cM apart are shown in greater detail (Fig. 1b). We observed significant LD between all marker pairs within 1 cM, 78% between 1 and 2 cM, 79% between 2 and 3 cM, and 39% between 3 and 4 cM (Table 2). Only 1.4% (12/842) of marker pairs greater than 4 cM apart show significant LD at the 0.01 level, slightly greater than that expected by chance alone.

We also measured LD using a *D'* statistic modified for multiallelic markers (Hedrick 1987). The rise in *D'* values for

Table 1. Genetic Map and Marker Statistics

Marker	Map position (cM)	H	Alleles observed ^a	Alleles analyzed ^b
S103	0.0	0.70	8	5
S117	0.5	0.87	15	8
S906	5.1	0.78	12	6
S193	9.1	0.72	13	4
S889	10.0	0.82	19	8
S482	11.8	0.69	9	4
S849	14.8	0.69	17	5
S905	18.6	0.64	11	4
S892	20.6	0.86	12	8
S846	20.6	0.73	10	5
S115	22.1	0.64	7	4
S851	25.5	0.76	14	6
S917	26.2	0.87	16	8
S894	30.4	0.83	12	7
S189	32.4	0.69	10	6
S898	37.3	0.74	18	5
S114	42.7	0.83	11	7
S912	48.2	0.83	12	7
S477	48.9	0.74	11	5
S874	52.1	0.79	16	6
S195	52.7	0.85	16	7
S909	53.4	0.70	16	5
S107	58.1	0.84	15	6
S170	59.6	0.80	17	8
S96	62.4	0.82	13	7
S119	65.7	0.80	10	6
S481	65.9	0.83	13	8
S836	66.9	0.83	12	7
S888	67.7	0.90	20	8
S886	68.1	0.86	10	7
S197	69.1	0.75	11	6
S178	70.2	0.78	11	7
S866	71.8	0.85	19	8
S196	74.5	0.79	23	4
S857	76.3	0.84	13	8
S480	79.2	0.75	11	6
S840	80.2	0.80	18	7
S211	80.2	0.65	13	5
S120	83.2	0.83	18	7
S100	85.8	0.74	12	5
S102	87.7	0.44	5	4
S171	96.1	0.80	13	6
S173	99.2	0.73	13	7

H, Heterozygosity.

^aNumber of alleles observed in an average of 5114 individuals per marker, typed as part of the FUSION study.

^bNumber analyzed when alleles with a frequency below 5% are pooled.

intermarker distances up to 4 cM is similar to the likelihood ratio *P*-values (Fig. 2). Importantly, this modified *D'* statistic is inversely related to sample size, as can be observed by comparing samples of 386 spouse controls and 837 affecteds to the combined sample. For distant marker pairs not expected to exhibit LD, the multiallelic *D'* statistic approaches asymptotes that are larger for smaller samples (Fig. 2).

The extent of LD depends on recombination rather than physical intermarker distance, although the presence of LD over kilobase distances can be used during positional cloning studies to determine the marker density needed to detect LD. We used finished and unfinished genomic sequence to estimate the physical distances between our markers. We evaluated the presence of significant LD for all 903 possible pairs of 43 markers, and show marker pairs up to 10 megabases (Mb)

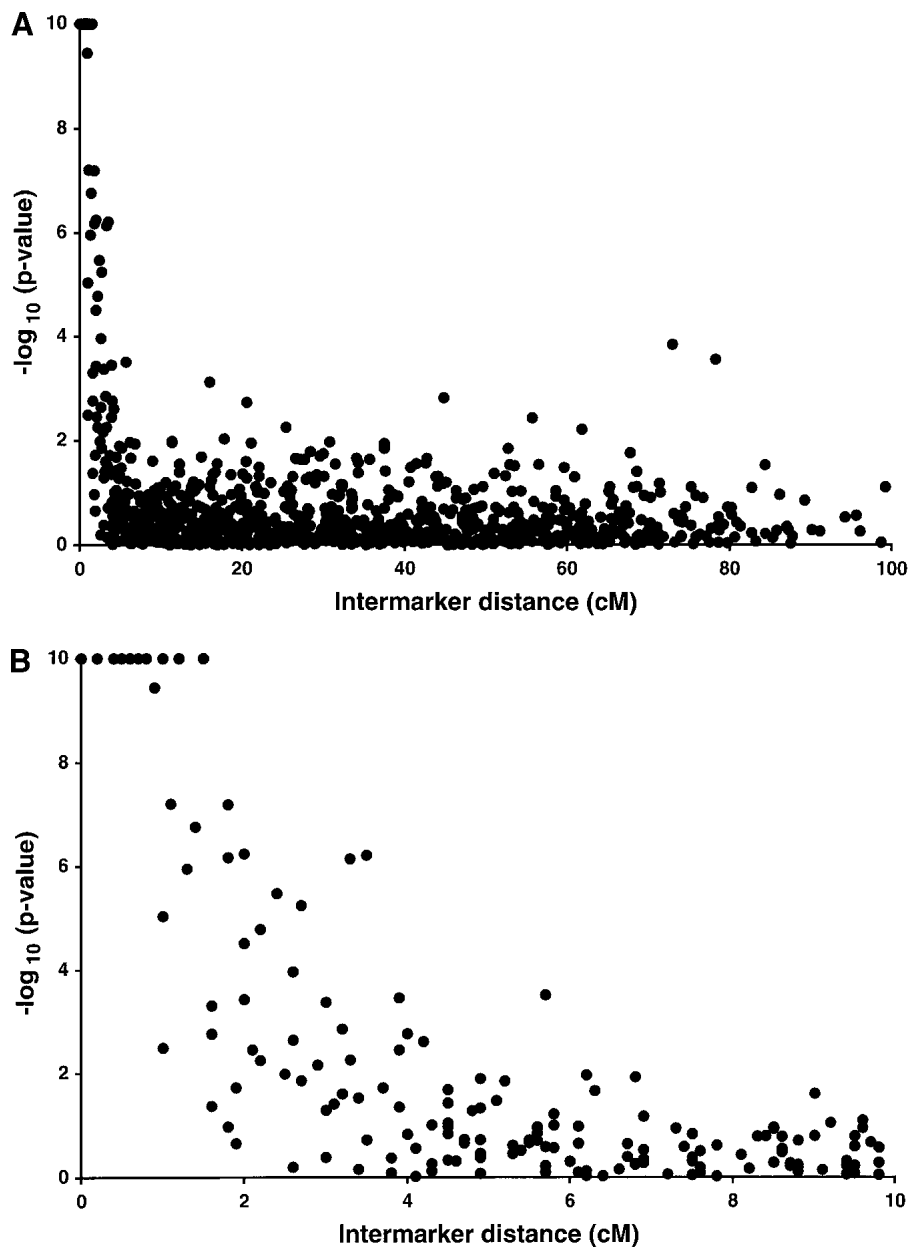


Figure 1 Presence of LD on chromosome 20 in 1223 Finns. $-\log_{10}(P\text{-values})$ for a likelihood ratio statistic, limited at 10^{-10} . Each point represents one pair of markers; (a) all 903 possible pairs of 43 markers, (b) 168 pairs of markers <10 cM apart.

apart (Fig. 3). Significant LD at the 0.01 level is observed for 69% (20/29) of markers up to 1 Mb apart, 51% (18/35) between 1 and 2 Mb apart, 12% (4/33) between 2 and 3 Mb apart, and 6% (2/36) between 3 and 4 Mb apart. Only 1.4% (11/770) of markers greater than 4 Mb apart showed significant LD. Strongly significant LD ($P < 10^{-10}$) was observed for all six marker pairs up to 350 kb apart, and 75% (9/12) for pairs up to 500 kb apart. As expected, the correlation between LD and physical distance (Spearman rank correlation $r = -0.56$) is less strong than with genetic distance ($r = -0.63$) for markers up to 10 cM apart.

the statistic is not valid for comparison with our data because it is strongly affected by sample size and allele frequencies. In general, smaller sample sizes show larger multiallelic D' values. At intermarker distances well beyond those where we would reasonably observe LD, we would expect the multiallelic D' values of different sized samples to approach the same asymptote. However, multiallelic D' values in our sample of 386 individuals approached an asymptote almost twice as large as that observed for 1223 individuals. We also investigated a recently reported statistic (Zhao et al. 1999) that has the advantage of providing a quantity that appears near mean

DISCUSSION

We analyzed marker-marker LD across all of chromosome 20 using a large sample of 1223 unrelated Finnish subjects. We observed significant ($P < 0.01$) marker-marker LD for all marker pairs within 1 cM of each other and continued elevated LD for distances up to 4 cM (Fig. 1). We obtained similar conclusions when analyzing the data based on significance level 0.001 (Table 2) or 0.05 (Fig. 1). We observed significant LD ($P < 0.01$) at estimated physical distances up to 4 Mb, with strongly significant LD ($P < 10^{-10}$) at distances up to 350 kb. We did not detect regions of chromosome 20 that exhibited obviously higher or lower LD (data not shown), although the variable spacing of our markers did not provide ideal data to address this issue.

Our findings of significant LD cannot be compared directly to results obtained from most other studies using microsatellite markers because the likelihood ratio test is strongly affected by sample size. In particular, our large sample size enables quite sensitive detection of LD. The expected value of the likelihood ratio statistic is proportional to sample size, while P -values are affected even more strongly by sample size. This characteristic is consistent with the smaller number of significant marker pairs observed in the smaller sample of 386 spouses (Table 2).

The likelihood ratio statistic shown here detects the presence but does not evaluate the strength of LD. To measure the strength of LD and to compare our data with a recent report (Eaves et al. 2000), we computed a multiallelic extension of the D' statistic (Fig. 2). Although this statistic is valid for the published comparison of samples with the same size that was typed using the same markers, we realized that

Table 2. Number of Marker Pairs in LD

Intermarker distance (cM)	n	$P < 0.01$			$P < 0.001$		
		spouses	affecteds	combined	spouses	affecteds	combined
<1 cM	11	11	11	11	10	11	11
1–2 cM	18	9	13	14	7	10	12
2–3 cM	14	2	8	11	1	4	7
3–4 cM	18	4	6	7	1	2	4
≥ 4 cM	842	17	16	12	0	3	4
Total in 99.2	903	43	54	55	19	30	38

P-values from a likelihood ratio test for LD. n, number of marker pairs. The spouse, affected, and combined samples consist of 386, 837 and 1223 individuals, respectively.

zero under the null hypothesis of linkage equilibrium; however, the variance of this statistic still depends on sample size. We are not aware of a good summary measure that enables accurate comparison of LD between studies that use different sample sizes and markers with differing numbers of alleles and differing allele frequencies.

We have previously reported evidence for linkage to type 2 diabetes on chromosome 20 in our Finnish sample (Ghosh et al. 1999). For LD analysis, one might expect heterogeneity when combining case and control samples. This would likely be true for a simple Mendelian disease in the region of the disease locus. However, for a complex disorder such as type 2 diabetes where a particular susceptibility variant alone likely has only limited independent impact on disease risk, we would expect little effect of susceptibility variants on LD along the chromosome. Thus, the similar extent of LD among individuals affected with diabetes compared to spouse controls (Table 2) and the homogeneity of haplotype frequency estimates between these groups is not surprising.

LD may depend on population substructure, which may be advantageous in the search for genes conferring susceptibility to a complex trait such as diabetes. Rare disease alleles in families from the north and east of Finland travel on haplotypes as large as 15 cM (de la Chapelle and Wright 1998), suggesting that it may be possible to identify diabetes-associated haplotypes in similar geographic subsets. Yet a re-

cent study found no difference between LD in 50 males from southwestern and eastern regions of Finland, founded ~2000 and ~400 years ago, respectively (Jorde et al. 2000). Two recent reports detected only modestly increased LD for the isolated Finnish and Sardinian populations compared to other European or American samples for microsatellites (Eaves et al. 2000) and single nucleotide polymorphisms (SNPs) (Taillon-Miller et al. 2000), respectively. Additional comparative data will help clarify whether LD is increased in various founder populations, enabling LD to be detected using smaller sample sizes or a lower density of markers.

Although methods to genotype SNPs are becoming easier and more accessible (Chen et al. 1998; Ryan et al. 1999; Griffin and Smith 2000), a high-density map of microsatellite markers remains extremely useful for studies of complex traits. The presence of multiple alleles allows haplotypes to be inferred more easily, generating more power to detect LD, although microsatellite marker mutation breaks down conserved haplotypes, and can also reduce the extent of LD. A significant challenge for assessing LD with microsatellite markers is the difficulty in generalizing LD statistics to multiple alleles, as described above.

We evaluated marker-to-marker LD as part of our investigation of disease variant-to-marker LD. Ideally, a threshold level of LD obtained between neighboring markers would indicate that the interval had been effectively scanned for an associated disease variant, although whether such a threshold

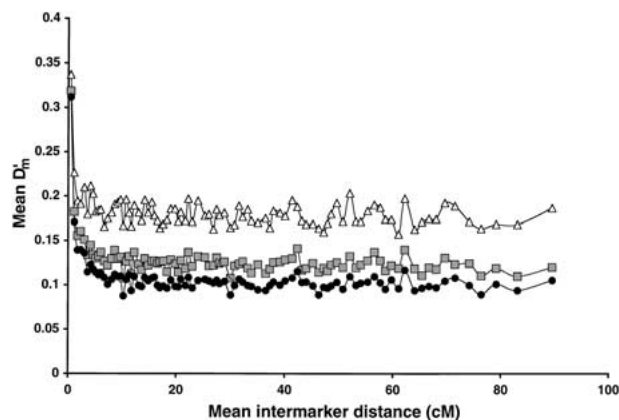


Figure 2 Strength of LD on chromosome 20 in 1223 Finns. Multiallelic D'_{th} statistic for 386 spouses (open triangles), 837 individuals with type 2 diabetes (shaded squares), and the combined sample of 1223 (black circles). Each point represents 10 marker pairs, and is plotted at the mean intermarker distance for those pairs.

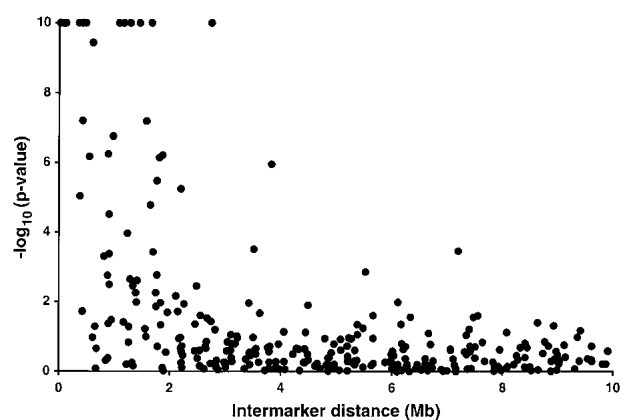


Figure 3 LD versus physical distance. $-\log_{10}(P\text{-value})$ for a likelihood ratio statistic, limited at 10^{-10} . Each point represents one pair of markers. Two hundred ninety marker pairs up to 10 Mb apart are shown.

can be found remains unclear. It seems highly likely that an interval lacking significant marker-to-marker LD has not yet been scanned thoroughly. Thus, to proceed with fine-mapping of a type 2 diabetes susceptibility gene on chromosome 20, we will add markers to regions where less marker-to-marker LD is observed while searching for alleles or haplotypes associated with disease. Additional empirical data are needed to determine the marker density required for association studies of complex diseases.

METHODS

Sample

The FUSION study design and first phase of sample recruitment have been described previously (Valle et al. 1998). Briefly, we sampled 580 families ascertained through a type 2 diabetes-affected sibling pair, and collected additional affected and unaffected relatives. A second cohort includes additional family members as well as a separate set of 275 families. All individuals studied are believed to be of Finnish heritage based on their birthplaces and their grandparents' birthplaces within Finland. In this report we used genotype data from 837 unrelated affected individuals and 386 unrelated spouses. Two hundred six of these spouses tested unaffected by oral glucose tolerance test (OGTT) in our study, 160 reported themselves to be unaffected, and 20 had an unknown phenotype.

Markers

We selected 43 microsatellite markers, including 39 dinucleotide and 4 tetranucleotide repeats, from chromosome 20 maps and genotyped them as previously described (Ghosh et al. 1997). We estimated heterozygosities and the number of alleles from an average of 1142 unrelated individuals per marker. These heterozygosities are very similar to those we reported previously (Ghosh et al. 1999), but differ slightly because we analyzed an expanded sample. Data for all markers are consistent with Hardy-Weinberg equilibrium.

Maps

We estimated sex-averaged genetic maps from combined data on 983 individuals in 205 FUSION-extended families and from cleaned genotypes of CEPH pedigrees (Broman et al. 1998). The genetic map used in these analyses differs slightly from a previously reported map (Ghosh et al. 1999) because additional FUSION data have now been incorporated. This marker order is consistent with the available mapping data produced by the Chromosome 20 Mapping Group at the Sanger Centre, and were obtained from the World Wide Web at <http://www.sanger.ac.uk/HGP/Chr20/>.

We estimated a physical map using chromosome 20 clone sequences available from the Sanger Centre and GenBank as of October 30, 2000. Most sequence data were produced by the human Chromosome 20 Sequencing Group at the Sanger Centre, and can be obtained from <ftp://ftp.sanger.ac.uk/pub/human/chr20/>. Other data were obtained from GenBank ftp://ncbi.nlm.nih.gov/genbank/genomes/H_sapiens/CHR_20/. We determined the nucleotide positions of our markers on individual clones using electronic PCR and BLAST (<http://www.ncbi.nlm.nih.gov/>), and used an unpublished clone order (P. Deloukas, pers. comm.) to generate a contiguous map. We assumed the size of six contig gaps to be 100 kb each, and the size of 12 unfinished BACs to be their current sequence length. We also assumed that 100 nucleotides of each clone overlaps the adjacent clone, and that all finished clones are numbered according to their orientation on the chromosome. The resulting physical map,

which starts at marker D20S103 and ends at marker D20S173, is 59 Mb long.

Tests of Linkage Equilibrium between Pairs of Markers

We formed 903 = (43*42)/2 marker pairs by considering all possible pairs of the 43 microsatellite markers. For each marker pair, we performed joint analyses for the 837 diabetic individuals and their 386 spouse controls. Maximum likelihood estimates for allele frequencies were obtained for each of the 43 markers by allele counting. Maximum likelihood estimates of haplotype frequencies for the 903 marker pairs were obtained by employing the expectation-maximization (E-M) algorithm. We used a likelihood ratio statistic to test for LD. This statistic compared the maximum probability of the observed sample of genotypes allowing for LD (based on estimates of haplotype frequencies) to the maximum probability of the observed sample assuming linkage equilibrium (based on estimates of haplotype frequencies calculated as products of the relevant allele frequency estimates). The distribution of -2 times the natural logarithm of this likelihood ratio is asymptotically distributed as χ^2 , with degrees of freedom equal to $(n_1 - 1)(n_2 - 1)$, where n_1 was the number of alleles used for the first marker, and n_2 was the number of alleles used for the second marker. To reduce the effects of rare alleles and to preserve the power to reject the null hypothesis of linkage equilibrium by limiting the degrees of freedom of the test statistic, we pooled alleles with frequencies less than 0.05.

Prior to pooling the data from the affected individuals and their spouses, we carried out a likelihood ratio test of homogeneity of haplotype frequencies for each pair of markers. For this test we estimated the haplotype frequencies separately for the affected individuals and their spouses using the E-M algorithm. The likelihood ratio statistic compared the product of the maximum likelihoods for the two samples to the maximum likelihood for the two samples analyzed jointly. Again, alleles with frequencies <0.05 in the joint sample were pooled. Because of small estimated haplotype frequencies, we assessed significance levels using a permutation test. For each of the 903 marker pairs, we generated 100 replicate samples by permutation. To construct these permuted samples, we randomly permuted affection status of the 1223 individuals, keeping the marker data the same. For each permuted sample, we calculated the likelihood ratio test for homogeneity of haplotype frequencies. Based on the 100 permuted samples for a marker pair, we estimated the P -value in the test for homogeneity of haplotype frequencies as the proportion of permuted-data statistics greater than the observed-data statistic.

To measure the strength of disequilibrium, we used Lewontin's D' (Lewontin 1964) modified for multiple alleles (Hedrick 1987). For two-allele markers, D' is the standardized disequilibrium value that takes the usual disequilibrium coefficient $P(A_i B_j) - P(A_i)P(B_j)$ and divides it by its maximal possible value. Given multiple alleles, we calculate the weighted average of the D' values where the weights are the products of the corresponding allele frequencies. That is,

$$D' = \frac{\sum_i \sum_j p_i q_j |D_{ij}'|}{\sum_i \sum_j p_i q_j}$$

where p_i and q_j are allele frequencies at the two loci of interest, and D_{ij}' is the standardized disequilibrium coefficient based on alleles A_i and B_j .

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