Analysis of a Detailed Genetic Linkage Map of *Lactuca sativa* (Lettuce) Constructed From RFLP and RAPD Markers

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ABSTRACT

A detailed genetic map has been constructed from the F_2 population of a single intraspecific cross of *Lactuca sativa* ($n = 9$). It comprises 319 loci, including 152 restriction fragment length polymorphism (RFLP), 130 random amplified polymorphic DNA (RAPD), 7 isozyme, 19 disease resistance, and 11 morphological markers. Thirteen major, four minor linkage groups and several unlinked markers are identified for this genome which is estimated to be approximately 1950 cM. RFLP and RAPD markers show similar distributions throughout the genome and identified similar levels of polymorphism. RAPD loci were much quicker to identify but more difficult to order. Procedures for generating accurate genetic maps and their limitations are described.

EARLY genetic maps of higher plants and animals were often generated from the analysis of many different, large populations each segregating for a few loci which were usually at best only loosely linked. These "hybrid" maps were time-consuming to generate and resulted in an average map for the species. With the advent of restriction fragment length polymorphism (RFLPs; BOTSTEIN *et al.* 1980) markers and more recently random amplified polymorphic DNAs (RAPDs; WILLIAMS *et al.* 1990), detailed genetic maps are being generated from single progenies of limited sizes (usually under 100 individuals). This raises issues of the applicability of the map to the species as a whole and the reliability and repeatability of the published gene orders. RFLPs have been used to identify the majority of loci in most of the current detailed maps; however, RAPD markers are being used with increasing frequency and their reliability as markers is being determined.

We have been developing a detailed genetic map of lettuce (*Lactuca sativa* L.) as part of our studies on the genetics of disease resistance. Interactions between diverse pathogens and plants have been shown to be determined by a gene-for-gene system (FLOR 1956; CRUTE 1985). Some of the most detailed genetic analyses of gene-for-gene interactions have been between *L. sativa* and the obligate biotrophic fungal pathogen, *Bremia lactucae*, the causal agent of lettuce downy mildew (CRUTE and JOHNSON 1976; JOHNSON *et al.* 1977; MICHELMORE *et al.* 1984; HULBERT and MICHELMORE 1985; FARRARA *et al.* 1987; ILOTT *et al.* 1989). We have now mapped 13 dominant genes for resistance to downy mildew (*Dm*) to four different linkage groups. These genes are clustered with genes for resistance to other pathogens and pests. We are currently locating many genes for disease resistance onto the genetic map of lettuce as precursors to molecular studies and manipulation in breeding programs.

Since the first gene was identified in lettuce (DURST 1929), many morphological, isozyme and disease resistance loci have been identified (for compilations, see ROBINSON *et al.* 1983; KESSELI and MICHELMORE 1986; MICHELMORE *et al.* 1993). However, prior to 1985, only four small linkage groups with 10 loci had been defined (LINDQVIST 1960; RYDER 1983). In this report, we describe the development of a detailed and accurate genetic map of *L. sativa* with several types of molecular markers and analyze the characteristics of the current map and the reliability and utility of its markers.

MATERIALS AND METHODS

Populations of *L. sativa*: The map was generated from the analysis of an F_2 population of an intraspecific cross between cv. Calmar and cv. Kordaat. Both parental cultivars are inbred lines. Calmar, the product of American breeding efforts, is a crisphead type. It possesses three genes for resistance to downy mildew (*Dm5/8, Dm7, Dm13*) and one gene for resistance to turnip mosaic virus (*Tu*). Kordaat, the product of European breeding programs, is a butterhead type. It possesses three different genes for resistance to downy mildew (*Dm1, Dm3, Dm4*). Early studies on isozyme diversity detected no less variation between these cultivars than between cultivated types and wild *L. serriola* (KESSELI and MICHELMORE 1986).

Sixty-six plants from the F_2 population were grown in the greenhouse and allowed to self-fertilize (LANDRY *et al.* 1987a). Both the plants of the F_2 and a bulk of 20 individuals from each F_3 family were used as sources of DNA. The segregation of dominant genes, such as resistance genes and morphological markers, was scored in F_3 families to determine the genotype of the progenitor F_2 plant. "Immortal" F_3 families were developed from each plant of the F_2 by growing 20 plants from each F_3 family and pooling the seed from each. Recombinant inbred lines are also being developed from each of the original 66

plants. The population has been expanded to 309 individuals to increase the accuracy of gene orders in some regions and to attach loosely linked loci to major groups.

Six crisphead type cultivars (Ithaca, Montello, Oswego, Salad Crisp, Vanguard and Vanguard75) and a wild *Lactuca saligna* (UCUS1) were used to examine the utility of RFLPs and RAPDs mapped in the 'Calmar' × 'Kordaat' cross, for comparative mapping in other crosses.

Disease resistance, isozyme and morphological genetic markers: Segregation of disease resistance genes was analyzed by inoculating with the appropriate isolate, 20 7-day-old seedlings for each F_3 family and scoring for the presence or absence of asexual sporulation as described by ILOTT *et al.* (1987). Each isolate expressed avirulence genes matching only one resistance gene (ILOTT *et al.* 1989). Isolate C83 M47 was used to detect *Dm1*. Isolate IM25R7 was used to detect *Dm3*. Isolate SF3 was used to detect *Dm4*. Isolate CG1 was used to detect *Dm5/8*. We have no isolate that detects only *Dm13*. Isolate CS7 detected *Dm3* and *Dm13*; therefore, the presence of *Dm13* could only be determined in those families shown by their reaction to isolate IM25R7 to lack *Dm3*.

Isozyme procedures were as described by KESSELI and MICHELMORE (1986). Compilations and descriptions of morphological markers are reported elsewhere (ROBINSON *et al.* 1983; MICHELMORE *et al.* 1993; O. OCHOA, R. V. KESSELI and R. W. MICHELMORE, manuscript in preparation).

DNA extraction and detection of RFLP markers: The extraction of genomic DNA, the construction of cDNA and genomic libraries as sources of probes for RFLPs, isolation and labeling of cloned inserts by nick translation, random primer techniques and genomic Southern hybridizations have been described previously (LANDRY *et al.* 1987a; KESSELI *et al.* 1991; PARAN *et al.* 1991).

The more than 2000 random cDNA and genomic DNA clones were labeled and hybridized to Southern blots of parental genomic DNA, digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII or *Msp*I to identify combinations of probe and endonuclease digest that detected polymorphism. Subsequently, probes were hybridized to blots of the DNA from the F_2 population, digested with the appropriate endonuclease. Blots were washed, exposed to film, strip-washed to remove the probe and rehybridized with additional probes from 6 to 15 times.

Random amplified polymorphic DNA (RAPD) markers: Procedures for detecting RAPD generally follow those of WILLIAMS *et al.* (1990); conditions were optimized by H. CHENG (UCD, now USDA/ARS, East Lansing, Michigan), L. BICKEL (UCD) and R. V. KESSELI. Stock solutions were: 1.25 mM each dNTP; 10 mM $MgCl_2$; 10 μM 10 mer oligonucleotide primer (Operon Technologies, Alameda, California, or University of California, Davis); 5 units/μl AmpliTaq DNA polymerase (Perkin-Elmer); and 10 × buffer containing 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin. After extraction, DNA concentrations were generally 0.4–0.5 μg/μl; therefore, this DNA was diluted, 1:100, with a modified TE (10 mM Tris, 0.1 mM EDTA) to give stocks of template DNA at approximately 5 ng/μl. Either 25- or 12.5-μl reaction volumes were used, overlaid with 2 drops of mineral oil. The 25-μl reaction volume comprised: 13.2 μl H_2O; 2.5 μl 10 × buffer; 2.0 μl dNTP stock; 1.1 μl $MgCl_2$; 1 μl primer; 0.2 μl Taq polymerase; 5.0 μl DNA. Polymerase chain reaction (PCR) was conducted in a Perkin-Elmer Cetus thermocycler with an initial period of 30 sec at 94° followed by 30–40 cycles of 94° for 1 min, 35° for 1 min, 72° for 2 min with a final extension at 72° for 5 min and a soak at 10° until the tubes were removed and refrigerated. About 2.5 μl of 10 × loading buffer (Ficol or sucrose based) was added under the oil, mixed and 13 μl of the mixture were then loaded onto 2.0% agarose TAE or TBE gels.

Sequence characterized amplified regions (SCARs): SCARs are PCR-based markers which identify a specific locus (PARAN and MICHELMORE 1992). SCARs are identified using a pair of oligonucleotide primers that, through PCR, amplify a single band. The sequence of these primers was derived from a band identified as a RAPD marker linked to a gene of interest. The RAPD fragment was cloned and partially sequenced. Then, 24 base oligonucleotide were synthesized (Operon Technologies) corresponding to the sequence at both ends of the fragment (the ten 5′ bases were the same as the original 10 mer used in the RAPD reaction to identify the fragment). These primers often amplified the alternate allele for the locus, thus providing a codominant marker.

Targeting markers to specific genomic regions: Several methods exist which could be used to improve the efficiency of mapping (MICHELMORE *et al.* 1992). We have used two methods to identify polymorphisms in specific regions while rapidly screening thousands of potential markers. The first method, comparing near isogenic lines (NILs), has been used in several species to target regions containing disease resistance genes (YOUNG *et al.* 1988; PARAN *et al.* 1991). Several NILs, products of backcross programs aimed at bringing resistance genes into desired cultivars, exist in lettuce.

A second method, bulk segregant analysis (BSA) has greatly increased both our mapping efficiency and our ability to target any region of the genome (MICHELMORE *et al.* 1991). This pooling procedure parallels the use of near isogenic lines, but can be implemented immediately for any region of the genome utilizing any population which segregates for the character or marker of interest. In its simplest form, the procedure involves separately pooling the DNA from individuals of each homozygote class for the target gene (*e.g.*, disease resistance or RFLP or RAPD marker at the end of a linkage group) and screening the two bulked samples for polymorphic markers (MICHELMORE *et al.* 1991). Only markers that are linked to the targeted gene will distinguish the bulks. We targeted intervals within a linkage group (gaps) in the same manner by pooling the two classes which are homozygous for markers flanking the region; individuals resulting from recombination within the region were excluded. Linkage was confirmed and gene order determined by conventional segregation analysis. These procedures allowed us to saturate specific regions of the genome containing resistance genes and coalesce previously unlinked sections of the genetic map.

Analysis of segregation data: The first map of lettuce (LANDRY *et al.* 1987a) was constructed by minimizing the recombination distance of all two point data generated from LINKAGE 1 (SUITER *et al.* 1983). The program provides recombination values with standard errors and tests (χ^2) segregation ratios and independent assortment of pairs of loci.

Later analyses relied mainly on the program MAPMAKER 2.0 (LANDER *et al.* 1987). This interactive program provides two point recombination values and multi-point ordering of loci based of user specified LOD scores. To detect linkage we used a threshold LOD score of 3.5 and a recombination frequency less than 0.3. To determine gene orders, we used a LOD score of 2; when a lower LOD had to be used to provide a final gene order, the region of uncertainty is bracketed. Recombination distances were converted to centiMorgans (cM) by the mapping function of Kosambi. In addition to the basic features of MAPMAKER, there are routines which dissect the data to uncover possible scoring and ordering errors as well as biologically interesting, aberrant loci and individuals. The "genotype" command displays individual genotypes within a linkage group and locates all recombination events as well as potential errors.

If a locus of an individual had a genotype different from both flanking loci, a recombination event on both sides of the locus would be required if the specified gene order was correct. When there are closely linked markers, this will be rare and therefore indicates a potential scoring error. The autoradiographs for RFLP loci and photographs of ethidium bromide stained gels for RAPD loci were all rescored with careful attention to verify all recombination events and inconsistent combinations of markers. Some locus-individual combinations were retested if the autoradiographs or photographs were questionable or inconclusive. Data points which remained uncertain were scored as missing. The whole data set was reanalyzed using the verified and corrected data.

The distribution of genetic markers along linkage groups was examined. Using a similar approach to that of BROWN and CLEGG (1983), we applied two tests from the ecological literature which analyze spatial distributions along transects. The first test, nonparametric nearest neighbor analysis (CLARK and EVANS 1954), examines all loci for "patchiness." Only loci which were identified by randomly selected probes and primers, and not those found through targeting procedures, were considered. Each linkage group was tested independently. The original analysis of CLARK and EVANS was for two-dimensional areas, not one- dimensional linear situations. The linear equivalent was derived both analytically and by computer simulation (B. JOHNSON, Department of Fish and Game, California). The assumptions of this analysis are that the density of markers is sparse and can be approximated by a Poisson distribution and there is independence of the nearest neighbor estimates. The second test was the non-parametric runs test (SOKAL and ROHLF 1981).

RESULTS

The genetic map of *L. sativa* now comprises 319 markers linked into 13 linkage groups of 4 or more markers (Figure 1) Of these markers, 152 are RFLPs, 130 are RAPDs and the remainder are genes for disease resistance (19), isozyme (7) and morphological characters (11). The estimated genome size is 1950 cM (derivations from HULBERT *et al.* 1988). If this estimate is correct, 98% of the new markers that we test should fall within 15 cM of a presently mapped marker (derivations from BISHOP *et al.* 1983). The map has yet to coalesce into nine chromosomal linkage groups indicating that there are many large gaps with few markers.

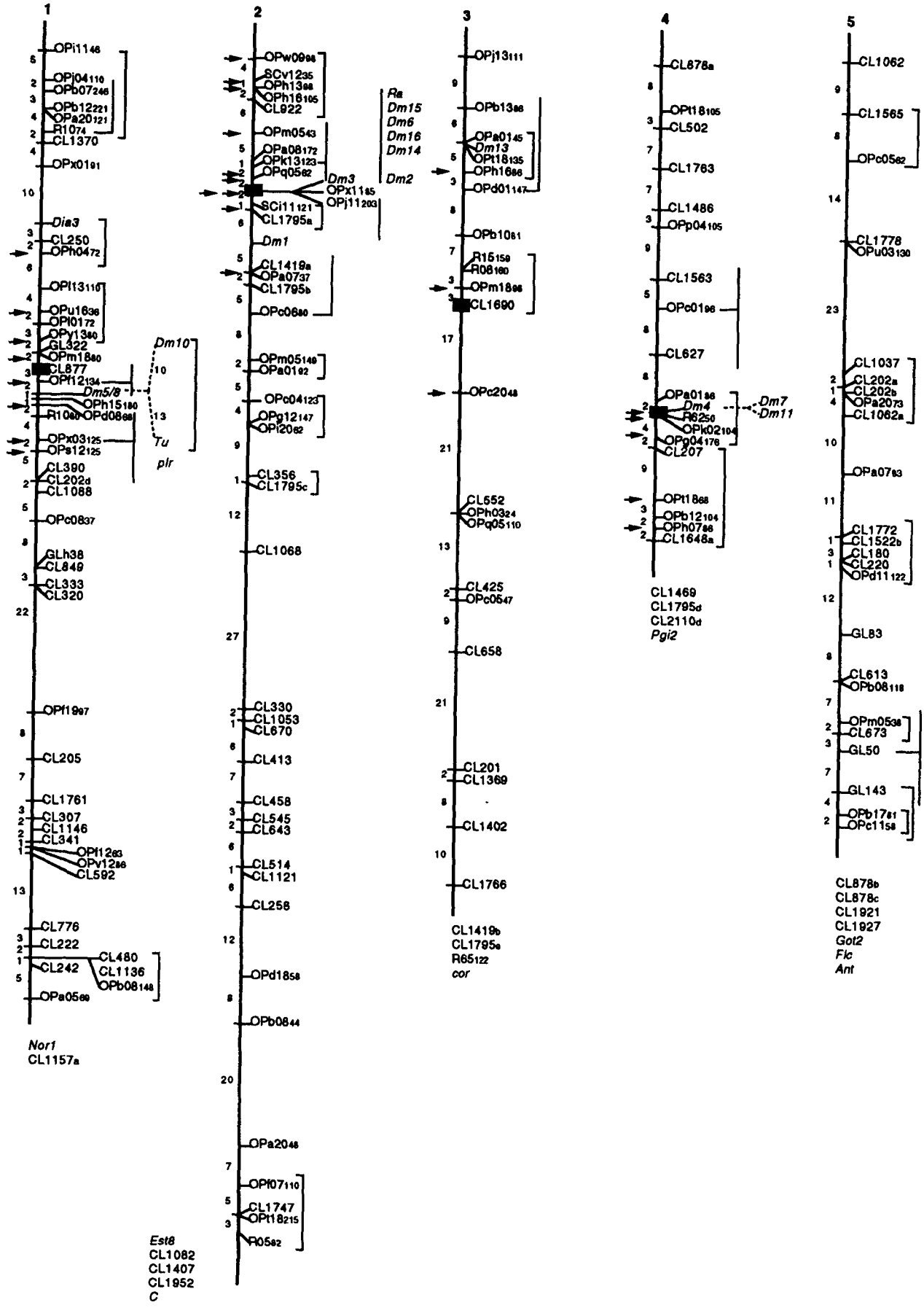
Copy number and sources of markers: While the majority of RFLPs were detected by cDNA clones, several libraries of random genomic fragments were also constructed and their efficiency at detecting low copy variability was examined. The genomic DNA for some libraries was digested with methylation insensitive enzymes *Mbo*I (LANDRY *et al.* 1987b), *Bam*HI and *Eco*RI (50 clones), and *Msp*I (36 clones). The efficiency of these was compared to libraries constructed with methylation sensitive enzymes, *Pst*I (319 clones), and *Hpa*II (78 clones). The methylation insensitive enzymes all yielded high frequencies (average = 0.93) of clones containing high copy DNA. With the methylation sensitive enzymes this frequency was reduced to 0.45–0.55 depending on the endonuclease and experiment.

About 11% of all RFLP clones (both cDNA and genomic clones) hybridized to two or more different size fragments in all digestions; these are considered as putative multigene families. Six clones identified multiple polymorphic loci. Two of these identified loci that were unlinked to each other (although other non-segregating bands could still represent linked loci). The others identified at least some members that are linked to each other. Two multigene families with 10 or more bands, had both linked and independent members. There was no evidence for large duplications in the genome as the different multigene families were not apparently linked to each other. However, there were regions which contained loci from several different multigene families but most of these families did not identify more that one segregating locus. Additional analyses in other crosses will be needed to map other loci of these multigene families and determine their complete distribution.

RAPD markers were assessed for the frequency of high copy sequences within the amplified fragment in a similar manner as genomic clones. Sixty single RAPD bands were excised from agarose gels, reamplified and rerun on agarose gels along with the original multibanded sample. Southern blots from these gels were hybridized to labeled total genomic DNA. Known low and high copy genomic clones were included as controls. About 70% of the bands showed hybridization to total genomic DNA and were presumed to contain at least some high copy sequences. Several of the putative low copy bands that showed no detectable hybridization to total genomic DNA, were labeled and hybridized to Southern blots of parental ('Calmar' and 'Kordaat') DNA in an effort convert dominant RAPD markers into codominant RFLP loci. The resulting hybridization patterns were always monomorphic between the parental lines and showed multiple bands in all five endonuclease digestions indicating that the amplified fragment was of low or moderate rather than single copy.

Levels of polymorphisms: The frequency of polymorphisms was low in our intraspecific cross. An analysis of a subset of the markers is shown in Table 1. The first 1008 cDNA clones that we examined detected 1107 putative loci (multiple bands were found with 10% of these probes). About 10% of these detected differences between our parental lines in at least one of the five endonuclease digestions. Of these polymorphisms, most could be scored unambiguously, segregated in the F_2 and were eventually mapped to yield 99 loci. The genomic clones detected similarly low levels. From an initial screen of 180 clones (*Pst*I library), 95 putative loci were identified (49% were low copy and 6 identified multiple loci). Of these, 11% detected differences between our parental lines and were eventually mapped to yield 10 loci.

The RAPD loci gave slightly lower levels of scorable polymorphisms. For the first 50 10-base primers tested, 426 scorable bands were amplified (averaged 8.5 bands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CL1795c
CL1068
CL330
CL1053
CL670
CL413
CL458
CL545
CL643
CL514
CL1121
CL258
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OPb0844
OPa2046
OPl07110
CL1747
OPt18215
R05a2
Est8
CL1082
CL1407
CL1952
C
Re
Dm15
Dm6
Dm16
Dm14
Dm2
3
OPj13111
OPb1388
OPa0145
Dm13
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CL1766
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CL1565
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CL1778
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CL202a
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OPa2073
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Ant

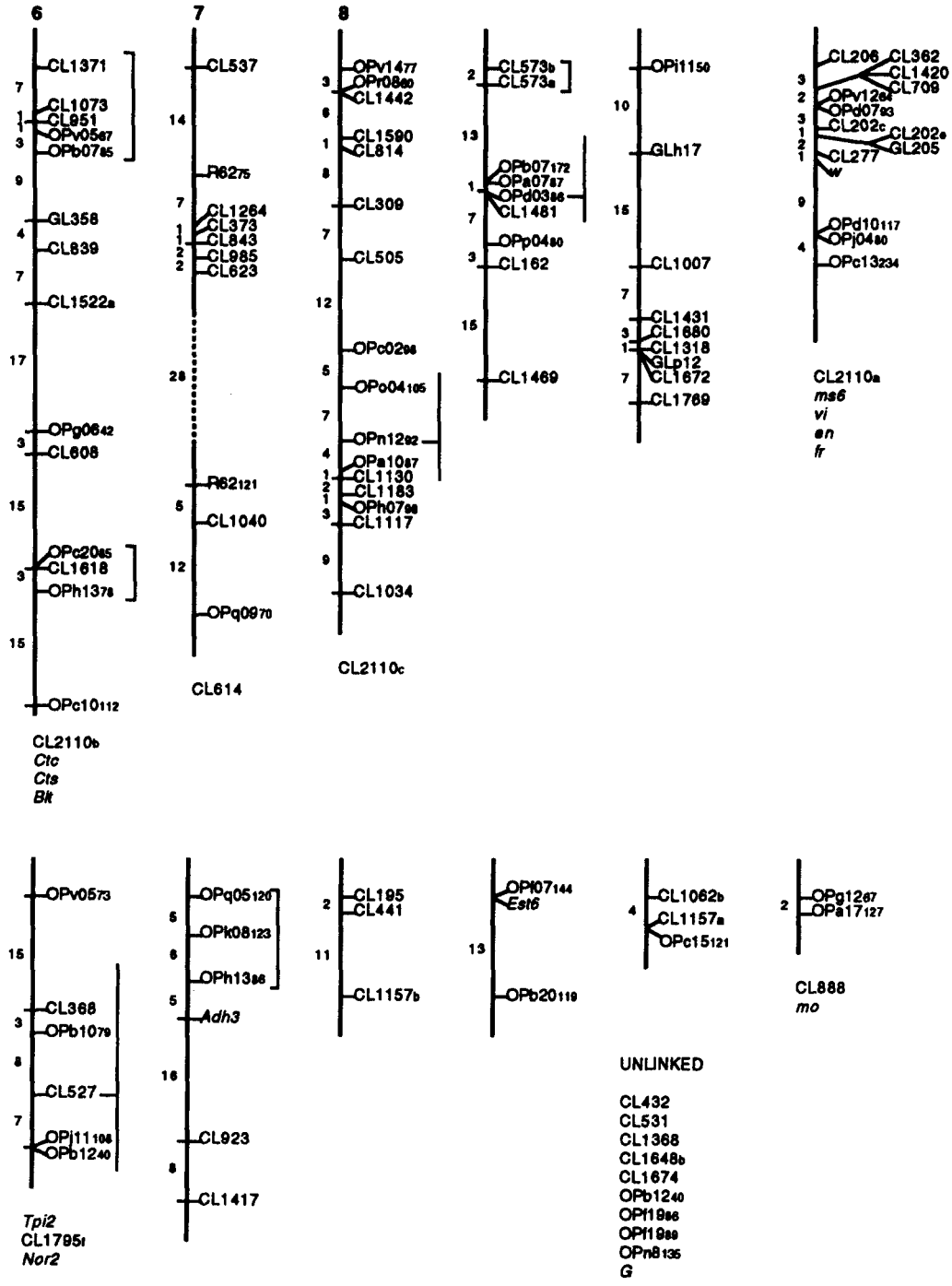

FIGURE 1.—Genetic map of *L. sativa*. The eight large linkage groups (>70 cM) are numbered at the top. Loci are listed to the right and recombination distances (cM) are listed to the left of each linkage group. Horizontal cross bars identify loci which are ordered at a LOD score of 3.0 or higher. Vertical brackets (for groups of loci) or "T" bars (for single loci) to the right of each linkage group indicate uncertain orderings with the best fit order only slightly better than the second alternative (LOD < 1.0). Markers at the base of linkage groups either could not be accurately placed on the group or were known from other studies to be linked to markers within the linkage group. The *CL-* and *GL-* prefixes indicate RFLP loci detected by cDNA and genomic DNA clones respectively. The *OP-* and *R-* prefixes indicate RAPD loci detected with Operon Technologies or UCDavis primers, respectively. Clusters of disease resistance genes are located to the right of linkage groups 1, 2 and 4 (a single resistance gene *Dm13* is located in group 3). These loci are: *Dm-*, resistance to lettuce downy mildew, *Ra*, resistance to root aphids and *Tu*, resistance to turnip mosaic virus. Other loci designations and descriptions follow notations in KESSELI *et al.* (1992) or MICHELMORE *et al.* (1993). Rectangular blocks indicate targeted loci and arrows indicate the loci identified by the use of either near isogenic lines (group 2 and 4) or bulk segregant analysis (group 1 and 3).

TABLE 1

Levels of polymorphisms for different types of molecular markers detected between two cultivars of lettuce (*Lactuca sativa*)

Type of marker	No. of probes or primers[a]	No. of loci	Proportion polymorphic	Proportion mapped
RFLP				
cDNA	1008	1107	0.10	0.09
Genomic	180	95	0.11	0.11
RAPD	50	426	0.17	0.07

[a] Subset of those used to generate the map.

per primer). Of these 17% appeared initially to show differences between our parental lines, but because of difficulties in scoring in the F_2, only 7% were eventually mapped (30 loci). These difficulties occurred because some bands were either faint or too close to other bands. The faint bands were sometimes mistaken as polymorphic but often were probably truly polymorphic but could not be scored reliably.

The data demonstrate the low level of variation that exists between even diverse plant types of cultivated lettuce. The comparisons show the relative efficiency of the different types of markers but absolute values depend on the specific cross used. For this intraspecific cross we averaged better than one polymorphism every two RAPD primers tested (30/50 in Table 1) and one in ten RFLP probes. With an interspecific cross *L. saligna* × *L. sativa*, the value was four loci per primer (R. V. KESSELI, unpublished). Similarly high levels of polymorphisms distinguish *L. saligna* and *L. sativa* at RFLP loci (KESSELI *et al.* 1991).

An odd feature of the RFLP data is that polymorphisms are rare (only 10% of the probes detect differences among these cultivars), yet when present, most (73%, see DISCUSSION and Figure 2) are detectable with multiple restriction endonucleases. This implies either a clustering of point mutations at restriction sites surrounding a few specific probes or, more likely, a few rare insertion/deletion events are the primary cause of detectable polymorphisms.

For both RFLP and RAPD loci, the utility of the genetic map in other crosses is restricted. To illustrate, 31 RAPDs and 31 RFLPs located on all eight major linkage groups were examined for their ability to detect polymorphisms in other crosses with crisphead types of lettuce (the economically more important group in the United States) and a wild *L. saligna* (Table 2). Most loci would segregate in 'Kordaat' × crisphead crosses but few would segregate in 'Calmar' × crisphead crosses since 'Calmar' is a crisphead. Three differences between RAPDs and RFLPs are notable. First, since RAPD loci showed just two alleles, a locus could be mapped in either a 'Calmar' × crisphead or 'Kordaat' × crisphead cross but not both; the sum of the loci mapped in the two crosses is always 31. The occasional multiple allelic locus allowed some RFLP loci to be mapped in both types of crosses. Second, RAPDs were more likely than RFLPs to segregate in 'Calmar' × crisphead crosses but less likely to segregate in 'Kordaat' × crisphead crosses. This was because loss of a band at a RAPD locus among crispheads was more common than a change of a fragment size at a RFLP locus. Third, RFLPs are superior

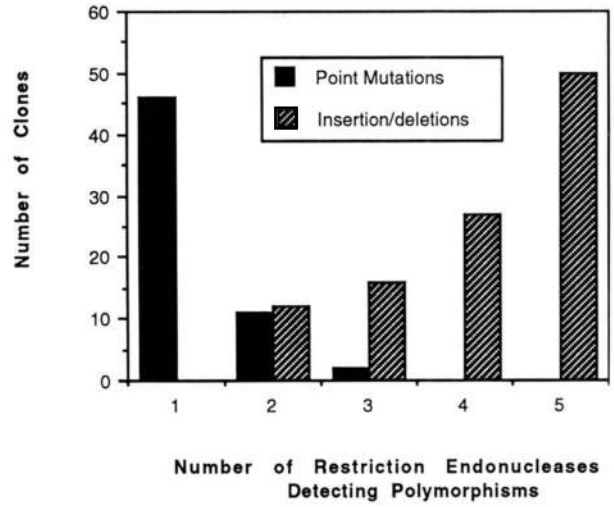

FIGURE 2.—Histogram showing the number of polymorphisms that appear to fit an insertion/deletion verses a point mutation expectation. From one to five restriction endonucleases detected polymorphisms with the 165 probes of this sample. Hashed bars indicate the number of clones that clearly showed a pattern indicative of insertion/deletions being the likely cause of detectable polymorphisms. That is, either all five restriction endonucleases detected a difference among the parental lines or if only a portion (2–4) of the restriction endonucleases showed a difference, the restriction endonucleases that produced the largest fragments were the polymorphic ones. The solid bars indicate the number of clones indicative of point mutation expectations. That is, differences were detected with only one of the five restriction endonucleases or there was no relationship between the size of detected fragments and their likelihood of being polymorphic.

TABLE 2

Ability of RAPDs and RFLPs, mapped in the standard crisphead × butterhead cross 'Calmar' × 'Kordaat' cross, to detect polymorphisms in other crosses involving one of these parents and each of six other crisphead cultivars or a wild species *L. saligna*

	Crisphead cultivars[a]						*L. saligna*
	Ith	Mont	Osw	Sal	Van	Van75	US1
RAPDs							
Calmar	7	5	9	7	12	11	17
Kordaat	24	26	22	24	19	20	14
RFLPs							
Calmar	5	5	6	6	9	8	31
Kordaat	27	27	26	26	25	25	31

Thirty-one loci were used for each type of marker and the body of the table identifies the number of loci, out of 31, that would segregate in a cross involving either 'Calmar' or 'Kordaat'.

[a] Cultivars were Ithaca, Montello, Oswego, Salad Crisp, Vanguard and Vanguard75. The *L. saligna* was UCUS1.

TABLE 3

Distribution of genetic markers along the eight large linkage groups of *Lactuca sativa*

Linkage group	Size (cM)	No. of loci	Nearest neighbor analysis (R)				Runs test (r)
			All loci	RFLPs	RAPDs	Resistance	
1	160	49	0.50**	0.46*	0.55	0.26*	17
2	201	44	0.54**	0.65	0.29**	0.19*	10*
3	147	23	0.48*	0.66	0.40*	NA	8*
4	82	19	0.69	0.90	0.82	0.00**	11
5	132	25	0.47*	0.61	0.75	NA	12
6	85	14	0.38*	0.63	0.42	NA	8
7	72	10	0.38	0.65	1.2	NA	6
8	71	16	0.55	0.46	0.42	NA	6

Nearest neighbor analysis: R is the ratio of the observed to the expected distance to the nearest neighbor for a specific type of genetic marker. A value of 1.0 indicates a random distribution. Smaller values indicate increasing levels of clustering. Larger values indicate a more regular distribution. Observed values are tested for significance by Z test.

Runs test: r is the number of runs of consecutive RFLP or RAPD markers obtained. Significance is based on the number of each type of marker in the linkage group (SOKAL AND ROHLF 1981). Markers used in these analyses do not include loci identified by targeting procedures.

* $P < 0.05$; ** $P < 0.01$; NA, not applicable.

when examining interspecific crosses. *L. saligna* always had an alternative allele at these loci, but for RAPDs, only half the loci, those with a "present" phenotype in the parental cultivar, could be mapped in any given cross (Table 2).

Segregation distortion: About 9% of the RFLP and RAPD loci showed segregation distortion (χ^2 goodness of fit). These markers mapped primarily to three regions of the genome. One region was the end of linkage group 2 and the distortion was caused by an excess of heterozygotes and deficiency of 'Calmar' homozygotes. The second region was in linkage group 7 which showed an excess of 'Kordaat' alleles at one end and an excess of heterozygotes at the other end. The final region was in linkage group 4 which showed a slight deficiency of 'Calmar' homozygotes.

We also detected five probes which were polymorphic in the parental lines but were monomorphic when tested in the F_2 and therefore could not be mapped. Two explanations are possible. The probes could be of plastid origin and therefore would be maternally inherited from the 'Calmar' parent, or there could be variation within the inbred parental lines and the specific individual used to produce the F_1 was different from what we now use as the parental stock. Although lettuce cultivars are highly inbred, several have been shown to be polymorphic mixtures of different homozygous genotypes (KESSELI *et al.* 1991).

Ordering the markers: Loci were assigned to a linkage group if the LOD scores for linkage were at least 3.5 and recombination frequencies were less than 0.3. The best possible orders are given in Figure 1. Brackets (for groups of loci) and "T" bars (for single loci) to the right of each linkage group indicate unresolved gene orders with the difference in LOD scores between the best and alternative orders of less than 2.0. Cross bars extending to the left of the vertical line on each linkage group identify the loci that form highly probable gene orders (LOD scores greater than 3.0). Groups of dominant loci were difficult to order precisely. Since the information provided by a pair of dominant, tightly linked loci in repulsion is nearly zero, loci linked in coupling were analyzed separately in some instances to produce two accurately ordered parallel maps anchored by codominant markers. These maps were then superimposed to produce the best fit map.

Clustering of loci: We have detected a non-random distribution of genetic markers on the recombination map of lettuce. RAPDs did not however, contribute more to this clustering then RFLPs. The data were analyzed using several statistical tests to determine the extent of the clustering. The first test, nonparametric nearest neighbor analysis (CLARK and EVANS 1954), examined the loci of a linkage group for "patchiness." The ratio of observed to expected distances, R, ranges from greater than 1.0 (regular or even distribution) through 1.0 (random distribution) to less than 1.0 (clustered distribution). Loci were analyzed in total or in separate classes (RFLPs, RAPDs or disease resistance genes) for each linkage group. Several large gaps, devoid of any genetic markers, were identified and significant clustering of loci was detected for five of eight linkage groups. Analyzed separately, RFLPs and RAPDs showed general clustering but only significantly so in three linkage groups (Table 3). A second analysis compared the relative distribution of RFLP and RAPD loci. The nonparametric runs test showed clustering in two of eight tests; a long run of RAPDs was found in only one region of the genome near *Dm13* (several marginally significant regions were also identified). These clustered RAPDs did not map to the exact same location but six of seven were amplified from the 'Kordaat' parent. This region may contain a series of short inverted repeats in the 'Kordaat' parent, could represent a large deletion in 'Calmar' not present in the cDNA library or could be deficient in the low copy sequences needed to detect RFLPs.

While the RFLP and RAPD loci each show minor levels of clustering, the disease resistance genes are highly clus-

tered. Fourteen of 18 mapped resistance genes are located in three groups covering approximately 35 cM. The classical map of the largest group (on linkage group 2) possesses eight disease resistance genes distributed over a 25 cM region. These have been ordered by classical genetic studies involving nearly 20,000 total progeny from 10 crosses screened with 10 different isolates of *B. lactucae* (T. NAKAHARA, R. W. MICHELMORE and R. V. KESSELI unpublished). This map has been superimposed onto our molecular marker map which segregated for two (*Dm1* and *Dm3*) of the eight resistance genes in this cluster.

Targeting regions to increase map density and bridge gaps: In previous studies we developed techniques to target markers to specific regions of the genome (MICHELMORE *et al.* 1991; PARAN *et al.* 1991). Here, we have identified nine additional markers in four regions by bulk segregant analysis (targeted loci for linkage group 1 and 3 are marked with rectangles and all loci identified by BSA are arrowed in Figure 1). One of these regions contains the one unclustered downy mildew resistance gene *Dm13*. This gene had been difficult to target since the locus requires combinations of isolates to score (see MATERIALS AND METHODS) and because its linkage group was small and remained unattached (KESSELI *et al.* 1990). With BSA, we targeted the only linked marker, the codominant RFLP *CL1690*, and successfully identified three RAPDs in the region. One of these, $OPC20_{48}$, bridged the gap and joined these loci to a larger linkage group (group 3 in Figure 1).

Increasing the accuracy of gene orders in the *Dm* regions: The map now contains many RFLP and RAPD markers within the three major *Dm* gene containing regions. We have increased the accuracy of the genetic map in these regions either by increasing the population size or by converting dominant markers to codominant ones. We generated a larger F_2 population from the original cross, 220 individuals (440 meioses). This allowed us to detect recombinants between loci that are less than 0.5 cM apart. In the region of the largest cluster of resistance genes (group 2 in Fig. 1), four RFLP loci (*CL922, CL1419, CL1795a* and *CL1795b*) and a RAPD marker, $OPW9_{98}$, were ordered with data from the expanded F_2 population. The order shown differs from the next best order by more than LOD 3.0. Without the increased population size four orders were within LOD of 3.0 of each other.

We converted some RAPD loci to codominant loci to overcome the difficulty in ordering dominant loci. Progeny testing of individuals with a RAPD fragment was done for a limited number of primers; this is slow and laborious as it requires F_3 individuals to be grown and many additional DNA extractions. Alternatively, locus specific SCAR primers were produced that sometimes identified the alternative allele (PARAN and MICHELMORE 1992). The termini of RAPD fragments were sequenced and two 24 base primers were synthesized. PCR with these primers at a higher annealing temperature to provide increased specificity, generally amplified a single band rather than the five to ten as with the 10 base RAPD primers. Some SCAR primers amplified a fragment from both parents. In most of these cases, the two alleles could be distinguished from each other either as length or restriction site polymorphisms. Two codominant SCARs, *SCv12* and *SCi11*, were identified in the region of the large cluster of disease resistance genes (group 2, Figure 1). They showed normal segregation and cosegregated with the dominant RAPD from which they were derived (PARAN and MICHELMORE 1992).

DISCUSSION

The genetic linkage map of lettuce now comprises 319 markers. There are eight large linkage groups of 70 cM or more in length, nine smaller linkage groups ranging from 2 to 41 cM, and 10 unlinked markers. The map has still not coalesced into the nine chromosomal linkage groups. With the population size used in this study, linkage could be reliably detected if markers showed less than 25–30% recombination (standard errors for pairs of codominant loci are 7–8%). Thus the gaps in the map are probably larger than 25 cM. A previous version of our genetic map (KESSELI *et al.* 1990) with 160 markers comprised eight large linkage groups and 19 pieces (smaller linkage groups and unlinked markers). Estimates for percent coverage (BISHOP *et al.* 1983) indicated that the 160 markers provided 85% coverage and that the map should have been saturated with the identification of 230 loci total. However, the additional 159 markers have not greatly reduced the number of linkage groups, although some previously unlinked pieces are now part of larger linkage groups.

All estimates for total genome size and percent coverage assume a random distribution of assayable markers over the genetic map. Nearly doubling the number of markers failed to reduce the number of linkages. This, plus the statistically significant clustering of loci within the boundaries of some large linkage groups indicated that the assumption of randomness has been violated in at least one of three ways. First, the parents and thus the progeny may be monomorphic for large, continuous stretches of the genome; potential markers may be distributed evenly along the map but they are undetectable. We are analyzing additional crosses to test this possibility. Second, there may be hotspots of recombination that introduce gaps into the genetic map. To bridge these gaps we shall analyze the segregation of terminal and unlinked markers in expanded F_2 populations as well as target by BSA markers to these regions as we successfully did for the *Dm13* region. Thirdly, there may be regions that lack readily assayable markers. Both RFLP and RAPD markers were utilized and the latter frequently detected polymorphisms in high copy DNA, but other

high copy and AT-rich regions are probably under sampled. Nucleolus organizing regions (NORs) may be responsible for some of the fragmentation in Lactuca. In the 'Calmar' × 'Kordaat' cross, no polymorphisms were detected with a ribosomal probe; in other crosses one NOR was at the end of a linkage group and the other was unlinked (unpublished data). Each gap of this sort may add 50 recombination units to the genome size and makes the resulting map look incomplete even though nearly 100% of new markers would link into preexisting linkage groups and coverage would be nearly complete. Physical mapping with addition lines or *in situ* hybridization may be the only way to tie together linkage groups spanning NORs or recombinagenetic gaps.

RAPDs are reliable, versatile and variable genetic markers that compare favorably with RFLPs. Strong bands that were well separated from other fragments and smaller than 2000 bp, identified RAPD loci that were misscored no more often than RFLP loci. Levels of polymorphism were also similar; slightly higher levels were probably present but more loci were discarded because of inconsistencies (see Table 1 and RESULTS). The majority of RAPD loci had present *vs.* absent phenotypes and therefore showed dominance; only three were codominant. This dominance decreased the accuracy with which RAPD loci could be ordered. In critical regions, however, the position of RAPD loci on the map were determined more accurately by increasing the population size, progeny testing, or developing codominant SCAR markers. The majority of RAPD fragments contained at least some high copy number sequences and therefore may be detecting polymorphisms in regions not accessible to RFLP analysis. However, RAPDs were generally no more clustered than RFLPs indicating that both types of markers are equally applicable to most genomic analyses. The main advantage of RAPDs over RFLPs and other types of markers such as simple sequence repeats (SSRs) lies in the large number of loci that can be screened quickly. In addition, RAPDs are particularly amenable to targeting techniques such as BSA. A disadvantage of RAPDs is that with a specific primer, the fragments amplified from one species are not generally homologous to those of another related species. All cultivars of lettuce show a similar amplification profile when using a given primer; most fragments are in common though polymorphic ones are present. Related species however, show completely different profiles. This precludes using RAPDs directly to do comparative genetic mapping in related species without an interspecific cross as a bridge. We have used SCARs to overcome partially this difficulty. Most SCAR primers, constructed from RAPD fragments identified from *L. sativa*, also amplify homologous sequences in other closely related *Lactuca* species (8 of 10 tested for *L. serriola* or *L. saligna*) and distantly related species (6 of 10 for *L. perennis* or *L. indica*). Some primers (3 of 10) have amplified homologous sequences in other members of the family Compositae such as chicory and sunflower (R. V. KESSELI, I. PARAN and R. W. MICHELMORE, unpublished).

Several characteristics of the lettuce genome made the mapping process difficult. As with many other species, the prevalence of high copy DNA is a major drawback. Non-methylation sensitive endonucleases yielded only about 10% low copy sequences. The methylation sensitive enzyme *Pst*I increased this to about 50%. These levels are lower than those obtain for most other plant species with available information (HELENTJARIS 1987; BURR *et al.* 1988; TANKSLEY *et al.* 1987; MILLER and TANKSLEY 1990a; APUYA *et al.* 1988; GEBHARDT *et al.* 1989; CHANG *et al.* 1988; SOLCUM *et al.* 1990). The advent of RAPD markers partially overcomes this mapping constraint. Simple sequence repeats (SSRs) may also be well suited to mapping studies in Lactuca as $(TG)_n$ and $(TC)_n$ repeats are found every 25–40 kb on average (R. V. KESSELI unpublished). A low level of intraspecific polymorphism also slowed the construction of the map. This level depends partly on the parental lines chosen and the number of digests observed, but is ultimately limited by the levels of variation present in sexually compatible taxa. The intra- rather than interspecific nature of the cross slowed the development of the map but yielded markers with greater utility with minimal segregation distortion. *L. serriola* is sexually compatible with *L. sativa*, but initial isozyme (KESSELI and MICHELMORE 1986) and later RFLP (KESSELI *et al.* 1991) surveys showed it to be only slightly more divergent than distant lines of *L. sativa*. We have begun analyzing a population from the interspecific cross between *L. sativa* and *L. saligna* (Table 2), but sterility and segregation distortion are high (R. V. KESSELI, O. OCHOA and R. W. MICHELMORE, unpublished).

Another characteristic of the lettuce genome may point to be the likely cause of polymorphisms. For the RFLP data, polymorphisms could be caused by point mutations at restriction sites, insertion/deletion events or other chromosomal rearrangements. We had noted earlier a correlation between the average fragment size detected with a specific endonuclease and the level of polymorphism (LANDRY *et al.* 1987b). MILLER and TANKSLEY (1990b) and MCCOUCH *et al.* (1988) noted that there was a correlation between any one endonuclease detecting a polymorphism and others detecting a polymorphism. These data imply that insertion/deletion events may be responsible for many polymorphisms. To characterize this, we examined the Southern blots of the parental DNA for 165 clones that had been analyzed with the same five endonucleases to determine whether the distribution was consistent with expected patterns obtained with both point and insertion/deletion mutation models. Assuming

that all mutations are independent events and that insertions are large enough to be detected as differences in all fragment sizes, then we expect that point mutations at restriction sites will be independent of the size of the fragment produced. Insertion/deletion events, however, cause changes for all restriction endonucleases with sites distal to the insertion but not between the insertion and the probe hybridization site. Thus, when a probe appears monomorphic with some restriction endonucleases and polymorphic with others, the monomorphic fragments should generally be smaller than polymorphic fragments. Approximately 70% of the RFLP variants appear to involve insertion/deletion or other complex events (Figure 2). This does not imply that insertion/deletions are more common than point mutations. Point mutations are only detected if they occur in the 12 base pairs of the restriction sites (assuming six base restriction endonucleases and no site within the region homologous to the probe). Insertion/deletions are detected if they are present anywhere between the restriction sites surrounding the region homologous to the probe (a length that can span 25 kb or more). Data involving the sequencing of specific alleles in Drosophila show that insertion/deletions and point mutations are both common. Most, but not all, of the large insertion/deletions in *Drosophila melanogaster* are caused by transposable elements (reviewed in AQUADRO 1992). Those data are difficult to compare with the current study since they are examining population data of sequence variation at specific loci and we are examining general patterns of variability across the genome for the two divergent types of *L. sativa*. Changes in genome size and major chromosomal rearrangements among related genera have been reported in plants (TANKSLEY *et al.* 1988) and we have reported the occurrence of recently duplicated sequences scattered in the genome of *Lactuca* spp. (PARAN *et al.* 1992). These kinds of complex changes may be common even in species with no documented transposable elements.

Gene orders were uncertain in several regions. The precise ordering of loci is constrained by statistical limitations. Assembling loci into linkage groups is increasingly easy as the number of loci rises; LOD scores of 3.5 or greater were used to assign loci to linkage groups. However, the precise gene order becomes progressively more difficult to determine as the number of informative meioses becomes limiting; "final" orders were often only slightly more likely than alternatives. Changes in population size make critical differences. In a population of 66 individuals, approximately 95% of the gametes will show no recombination in a 5 cM region and provide no information on order. Orders in the region will be based on few individuals from the whole population, in our case about 6 or 7 plants. The problem is exacerbated with dominant loci, such as RAPDs, which at close distances in coupling conceal half the recombination events (the mean information supplied by an individual is half that of codominant markers; ALLARD 1956). The mean information for dominant loci in repulsion is essentially zero when loci are tightly linked. Thus, in the absence of codominant markers (RFLPs), dominant markers are very difficult or impossible to order unless population sizes are large. For example in the *Dm13* region of our genetic map, there are two codominant markers, RFLP *Cl1690* and *Dm13* (after progeny testing), and 11 dominant RAPDs. One RAPD, *OPd11*, is in repulsion to the others and maps with near equal likelihood (LOD < 1) to eight possible positions. The RAPDs in coupling also do not precisely align, thus the total number of potential orders in the region which are acceptable at LOD > 2 is more than 20.

In the map presented here, we have attempted to resolve ordering ambiguities. It is not, however, critical to order all loci precisely. We are now assigning loci to "bins" and only determining gene order in regions of particular interest, usually flanking disease resistance genes. We are continuing to employ several strategies to resolve mapping ambiguities in these regions. The population size of the F_2 is increased and only those individuals that are recombinant in a particular region are analyzed (KESSELI *et al.* 1994). Critical, dominant loci are ordered either by analyzing individuals within F_3 families or by creating codominant SCARs from specific primers.

One feature which hampers all aspects of map development, particularly ordering, is experimental error such as misscored individuals or contamination. Errors inflate the number of apparent recombinants and expand map distances; this is especially severe when markers are tightly linked. If a misscored marker is located near the end of a group, the most likely order often places it in the terminal position. If the misscored locus resides internally, it may get placed at or near the correct position but will cause a considerable expansion of the map. Several features of MAPMAKER (*e.g.*, the "genotype" command) aid in the identification of flanking crossovers and possibly misscored individuals or loci. In this study, all data sets were carefully rescored and reanalyzed. All questionable data points were retested. This limited the experimental error. Of the more than 20,000 data points, 73 were found to be misscored. The morphological and resistance gene loci were the most error prone. For example, the original analysis placed *Dm4* at the end of a linkage group well separated from other markers (LANDRY *et al.* 1987a). Rescoring eight suspect F_3 families placed this gene within a cluster of markers. RFLP and RAPD markers were misscored at about equal frequency. The RFLP errors tended to be caused by small regions of Southern blots that transferred incompletely or by background signals obscuring

an individual band. The RAPD errors were usually in loci identified by large fragments (> 1.5 kb). The amplification of fragments of this size appeared more sensitive to the concentration of template DNA and primer in the reaction; relatively high levels of primer caused poorer amplification of larger but better amplification of smaller fragments.

Combining information from multiple crosses is necessary to map genes that do not segregate in the original mapping population. Generating many detailed maps from different crosses is neither necessary nor practical. A more efficient approach is to develop a detailed standard map from one or a few crosses and then superimpose small detailed regions generated from other crosses onto the standard map. With bulked segregant analysis, we identify markers linked to the target gene of the new segregating populations. Many of these markers also segregate in our standard mapping population. This quickly places the target gene on the map and simultaneously identifies additional linked markers in the standard population that are subsequently analyzed in the new cross to locate the gene precisely. This yields a consensus map for the species as more data from different crosses are combined; it may also identify regions in which gene orders are not conserved among lines. We have successfully located the disease resistance gene *plr* to a linkage group using this procedure (KESSELI *et al.* 1994) and are currently determining the location of others (*mo* and *cor*, our unpublished data).

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