

# Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations

(random amplified polymorphic DNA/restriction fragment length polymorphism)

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Communicated by Charles M. Rick, July 12, 1991 (received for review March 27, 1991)

**ABSTRACT** We developed bulked segregant analysis as a method for rapidly identifying markers linked to any specific gene or genomic region. Two bulked DNA samples are generated from a segregating population from a single cross. Each pool, or bulk, contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The two bulks are therefore genetically dissimilar in the selected region but seemingly heterozygous at all other regions. The two bulks can be made for any genomic region and from any segregating population. The bulks are screened for differences using restriction fragment length polymorphism probes or random amplified polymorphic DNA primers. We have used bulked segregant analysis to identify three random amplified polymorphic DNA markers in lettuce linked to a gene for resistance to downy mildew. We showed that markers can be reliably identified in a 25-centimorgan window on either side of the targeted locus. Bulked segregant analysis has several advantages over the use of near-isogenic lines to identify markers in specific regions of the genome. Genetic walking will be possible by multiple rounds of bulked segregation analysis; each new pair of bulks will differ at a locus identified in the previous round of analysis. This approach will have widespread application both in those species where selfing is possible and in those that are obligatorily outbreeding.

We have developed a method, bulked segregant analysis, as a rapid procedure for identifying markers in specific regions of the genome. The method involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool, or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. Two pools contrasting for a trait (e.g., resistant and susceptible to a particular disease) are analyzed to identify markers that distinguish them. Markers that are polymorphic between the pools will be genetically linked to the loci determining the trait used to construct the pools. Bulked segregant analysis has two immediate applications in developing genetic maps. Detailed genetic maps for many species (1) are being developed by analyzing the segregation of randomly selected molecular markers in single populations. As a genetic map approaches saturation, the continued mapping of polymorphisms detected by arbitrarily selected markers becomes progressively less efficient (2). Bulked segregant analysis provides a method to focus on regions of interest or areas sparsely populated with markers. Also, bulked segregant analysis is a method for rapidly locating genes that do not segregate in populations initially used to generate the genetic map.

Two types of molecular markers have been used to develop detailed genetic maps, restriction fragment length polymor-

phisms (RFLPs) and random amplified polymorphic DNAs (RAPDs). The majority of studies over the last 7 years have employed RFLPs. They are often codominant but are restricted to regions with low or single copy sequences. Recently, RAPD markers have been developed by Williams *et al.* (3). This technique relies on the differential enzymic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers (usually 10-mers). Polymorphisms result from either chromosomal changes in the amplified regions or base changes that alter primer binding. The procedure is rapid, requires only small amounts of DNA, which need not be of high quality, and involves no radioactivity. As no Southern hybridization is involved, polymorphisms can be detected in fragments containing highly repeated sequences; this provides markers in regions of the genome previously inaccessible to analysis. The RAPD markers are usually dominant because polymorphisms are detected as the presence or absence of bands. RAPD markers provide a quick method for generating genetic maps and analyzing populations. The ability to target RAPD markers efficiently to specific genes or regions monomorphic for previously characterized markers or to regions sparsely populated with markers will increase their usefulness further.

In this paper, we describe bulked segregant analysis as a method for rapidly identifying RFLP or RAPD markers in any genomic region of interest. We illustrate the procedure by identifying RAPD markers linked to a disease-resistance gene for which no near-isogenic lines (NILs) exist and by analyzing the performance of RFLP and RAPD markers that are known to be linked to disease-resistance genes. This procedure efficiently identifies markers linked to genes of interest, allowing their rapid placement on a genetic map. It also can be used to consolidate genetic maps by identifying markers in sparsely populated regions and at the end of linkage groups.

## MATERIALS AND METHODS

**Basic Method.** Bulked segregant analysis involves screening for differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Each pool, or bulk, contains individuals selected to have identical genotypes for a particular genomic region ("target locus or region") but random genotypes at loci unlinked to the selected region (Fig. 1). Therefore, the two resultant bulked DNA samples differ genetically only in the selected region and are seemingly heterozygous and monomorphic for all other regions. The two bulks are screened for differences the same way as NILs, with several RFLP probes simultaneously (4) or individual RAPD primers (5–10 loci

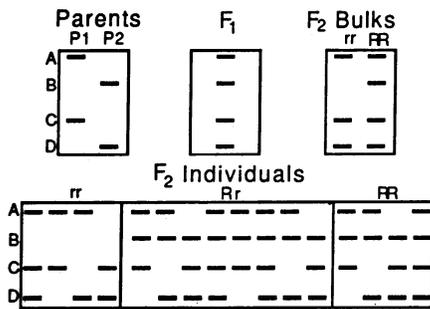


FIG. 1. Genetic basis of bulked segregant analysis. The schematic shows genotypes of four RAPD loci (A–D) detected by a single primer in two parents (P1 and P2), their F<sub>1</sub> and F<sub>2</sub> progeny, and bulks derived from F<sub>2</sub> individuals homozygous for resistance or susceptibility. The dominant allele at locus B is linked in cis to the R allele and therefore is polymorphic between the bulks. The other three loci that are polymorphic between the parents are unlinked to the resistance locus and therefore appear monomorphic between the bulks. This is an interpretation of the pattern obtained with primer OPF12 in Fig. 4.

assayed per primer). Of the two types of markers, RAPD primers provide the most efficient way of identifying new loci. However, many markers on existing maps rely on RFLP analysis. Linkage between a polymorphic marker and the target locus is confirmed and quantified by using the segregating population from which the bulks were generated. Probes or primers for loci that are polymorphic and absolutely linked to the gene or region used to distinguish the individuals comprising the bulks will detect clear differences between the bulks. In contrast, unlinked loci will appear heterozygous with approximately equal band intensities in each bulk. Recombination between the target marker and the assayed polymorphic locus will result in diminishing distinction between the two bulks with decreasing linkage until the locus appears unlinked. Obviously, loci not segregating in the population, whether linked or not, will not distinguish the bulks. Bulk segregant analysis does not reveal novel types of variation but rather allows the rapid screening of many loci and therefore the identification of segregating markers in the target region.

**Plant Material.** To identify new markers linked to *Dm5/8* and analyze the performance of existing markers, bulks were made from the basic mapping population, cvs. Calmar × Kordaat, used to generate the genetic map of lettuce (5, 6). This F<sub>2</sub> population consists of 66 individuals and segregates for six downy mildew resistance genes, *Dm1*, *Dm3*, *Dm4*, *Dm5/8*, *Dm7*, and *Dm13*. DNA was extracted as described (5) or by a modified hexadecyltrimethylammonium bromide (CTAB) procedure (7). Aliquots (2.5 μg of DNA) of each individual homozygous for one or the other allele of the targeted gene were bulked together. The number of individuals in each bulk varied between 14 and 20 plants. The bulks were screened with arbitrary RAPD primers. Linkage between *Dm5/8* and loci polymorphic between the bulks was confirmed and quantified by analyzing their cosegregation in the F<sub>2</sub> population used to construct the bulks. The F<sub>2</sub> population of a second cross, *Lactuca sativa* cv. Saffier × *Lactuca serriola* PIVT1309, comprising 80 individuals, was analyzed for markers linked to *Dm16*.

A F<sub>2</sub> population was used, as it provided the greatest genetic window (the segment of the genome in which markers are likely to be detected) around the locus. F<sub>3</sub> families from each F<sub>2</sub> individual had previously been analyzed for resistance (5). F<sub>2</sub> individuals heterozygous for *Dm5/8* were excluded from the analysis to allow the identification of RAPD markers from both parents (i.e., RAPD bands in both cis and trans to the dominant *Dm5/8* allele). If F<sub>3</sub> analysis had not

been available, the homozygous dominant and heterozygous genotypes could not have been distinguished at the F<sub>2</sub> level and would have had to have been bulked together; therefore, only RAPD bands associated in cis with the dominant *Dm5/8* allele could have been detected.

**RFLP and RAPD Markers.** Procedures for RFLP analysis were as described (5, 8). RAPD markers were amplified by using single oligonucleotide primers and DNA from the bulks and their parents as templates. The majority of the 10-mer oligonucleotides were from the commercially available RAPD primer kits A to F (Operon Technologies, Alameda, CA); these are designated by an "OP" prefix and then the kit letter and primer number. The reaction conditions were as described by Williams *et al.* (3) and Paran *et al.* (8). Approximately 20 ng of DNA was used as a template in a 25-μl reaction volume.

**Linkage Analyses.** New RAPD markers were mapped by analyzing one of the two F<sub>2</sub> populations with the program MAPMAKER (9). Linkage was considered significant if the logarithm of odds (lod) score was ≥3.0. For consideration of the positions of RFLP or RAPD loci relative to the target locus, linkage distances were calculated as two-point data. The distances shown on the genetic map were calculated from multipoint analyses.

## RESULTS

**Determinations of Sensitivity.** Bulk segregant analysis assumes that markers adjacent to the targeted gene will be in linkage disequilibrium (i.e., recombination will not have randomized these markers with respect to the gene). As the linkage distance increases, more recombinants will be present in each bulk, culminating in 50% recombinants, no linkage disequilibrium, and therefore no differences between the bulks. The sensitivity of bulk segregant analysis to detect unequal amounts of alleles was tested with RFLP and RAPD markers to determine at what genetic distance there would be sufficient recombinants to cause the bulked samples to appear monomorphic. Both the screening of artificial mixtures of parental DNA with single markers and the screening of bulks with markers known to be linked to various degrees with the target loci were tested.

Artificial mixtures were used to test the sensitivity of RAPD and RFLP analysis. For RAPDs, DNA samples from two species, *Lactuca saligna* and *L. sativa*, were mixed in various ratios. The use of distinct species ensured a large number of polymorphic bands. Reciprocal dilutions were made to provide a series of 15 mixtures with each species present in proportions of 0.0, 0.001, 0.02, 0.04, 0.1, 0.2, 0.4, and 0.5. These mixtures were screened for RAPDs with an arbitrary primer (Fig. 2; 0.04–0.001 dilutions are not shown). As anticipated, the precise sensitivity of the procedure to detect the rarer allele was band (locus) specific. However, RAPD analysis was unexpectedly insensitive in its ability to detect the rarer allele. The rarer allele was barely detectable at a proportion of 0.1 of the mixture and was never detected if it constituted a proportion of 0.04 or less of the total. Depending on the band, polymorphisms could be detected when the rarer allele constituted proportions up to 0.2–0.4 of the mixture, at least as differences in band intensities. Therefore, segregating markers within a window of 10% recombination either side of the target locus will always be detectable. Many markers within a 30% recombination window will also be detectable, at least as bands of unequal intensity. Similar experiments were done with a RFLP marker (*CL922*; data not shown). Again, in mixtures equivalent to 20% or less recombination, linkage could be detected because the hybridization intensities between the alleles was obviously different. In mixtures equivalent to 30% recombina-

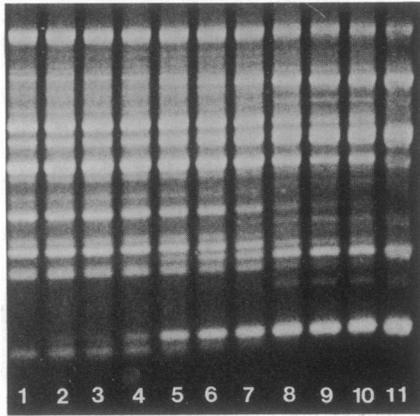


FIG. 2. Ethidium bromide-stained gel demonstrating the sensitivity of RAPD analysis to detect rare alleles. DNA from *L. saligna* and *L. sativa* was mixed in various ratios and used as substrate for amplification with a 10-mer oligonucleotide primer (OPD03). The proportion of DNA from *L. sativa* cv. Vanguard 75 was 1.0 (lane 1), 0.9 (lane 2), 0.8 (lanes 3 and 4), 0.6 (lane 5), 0.5 (lane 6), 0.4 (lane 7), 0.2 (lanes 8 and 9), 0.1 (lane 10), and 0.0 (1.0 *L. saligna*; lane 11). Bands unique to one parent show varying persistence as their prevalence decreases.

nation or greater, the hybridization intensity of the two alleles was indistinguishable.

To characterize the sensitivity further, bulks differing for disease resistance were made and analyzed with markers known to be linked to the resistance gene. To test for sensitivity with RFLP markers, DNA was bulked from  $F_2$  individuals homozygous for alternate alleles of *Dm16*. *CL922* absolutely cosegregates with *Dm16* in the 80 progeny so far analyzed. Hybridization with pCL922 revealed a clear poly-

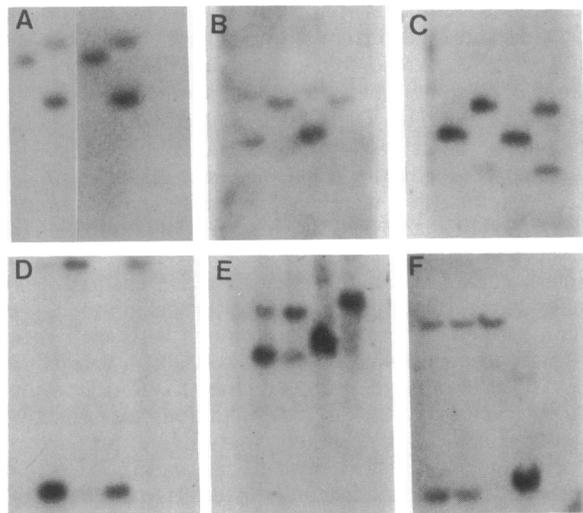


FIG. 3. Southern blots of parental and bulked DNA samples probed with markers at known distances from the loci used to distinguish the bulks. (A–C) Bulks were made for alternate alleles of *Dm16* from  $F_2$  individuals of *L. sativa* cv. Saffier  $\times$  *L. serriola* PIVT1309. (D–F) Bulks were made for alternate alleles of *Dm5/8* from  $F_2$  individuals of cvs. Calmar  $\times$  Kordaas. The first lane contains bulked DNA from homozygous-susceptible individuals; the second lane contains DNA from homozygous-resistant individuals. The third and fourth lanes contain parental DNA from PIVT1309 and Saffier, respectively (A–C), or Kordaas and Calmar, respectively (D–F). Blots in A, B, and C were probed with pCL922, pCL1419, and pCL1407, respectively. *Dm16* is 0 cM, 7 cM, and 9 cM from *CL922*, *CL1419*, and *CL1407*, respectively. Blots in D, E, and F were probed with pCL250, pCL849, and pCL1007, respectively. *Dm5/8* is 11 cM from *CL250*, 26 cM from *CL849*, and unlinked to *CL1007*.

Table 1. The sensitivity of known RAPD markers to detect polymorphism between bulked segregants

Primer	<i>Dm</i> gene targeted	Recombination frequency	Map distance	Polymorphic
OP111	3	0.02	2	Yes
R62	4	0.13	15	Yes
OPB12	4	0.21	27	No
OPA01	3	0.25	35	No

morphism between the bulks comparable to that between the parents; markers 7 and 9 centimorgans (cM) from *Dm16* were also clearly polymorphic between the bulks (Fig. 3).  $F_2$  bulks were also made for *Dm5/8*. Both alleles were detectable for a marker 11 cM from *Dm5/8* but in obviously nonstoichiometric amounts. At 26 cM the nonstoichiometry of the alleles was detectable but not obvious. This marker would not have been selected as distinguishing the bulks. The unlinked marker appeared heterozygous. To test for sensitivity of bulked segregant analysis with known RAPD markers, bulks were made from  $F_2$  individuals differing at *Dm4* or *Dm3*. RAPD markers <15 cM from *Dm4* were polymorphic between the bulks, whereas markers at least 27 cM away were monomorphic (Table 1). Therefore, the sensitivity of bulked segregant analysis seems to be approximately the same with RFLP and RAPD markers. All markers closer than 15 cM are likely to be detected; the limit of detection seems to be  $\approx$ 25 cM.

**Selection of Markers Linked to *Dm5/8*.** Bulked segregant analysis was employed to identify RAPD markers linked to *Dm5/8* for which no NILs exist. Two bulks were made, each of 17  $F_2$  individuals homozygous for alternate alleles of *Dm5/8*. Homozygotes had been identified by prior  $F_3$  family analysis (5). The pair of bulks was screened for RAPDs by using 100 arbitrary primers. The primers detected an average of nine bands per primer. Three primers, OPF12, OPH04, and OPH15, generated amplification products that were present in one bulk but not in the other. The polymorphism was confirmed by a repeated amplification and compared with the two parents (Fig. 4). The polymorphisms were mapped by

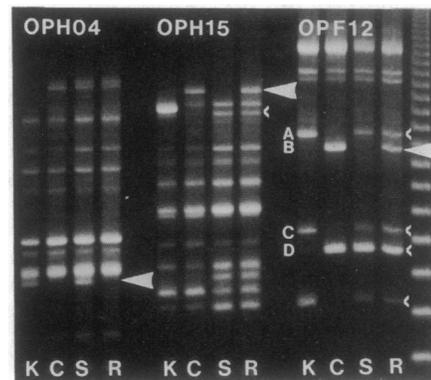


FIG. 4. RAPD markers detecting polymorphisms between bulks made for alternate alleles of *Dm5/8*. Each set of four lanes results from PCR amplification with a different 10-mer oligonucleotide primer: OPH04, OPH15, or OPF12. In each set, the first and second lanes contain parental DNA from Kordaas (K) and Calmar (C). The third lane contains bulked DNA from the homozygous susceptible individuals (S), and the fourth lane contains bulked DNA from the homozygous resistant individuals (R). The polymorphisms distinguishing the bulks are indicated by a solid arrowhead. Other polymorphisms at unlinked loci that distinguish the parents but not the bulks of  $F_2$  individuals are indicated by small open arrowheads. Occasionally bands are present in the bulks but not either parent; this phenomenon is sometimes also observed with heterozygous individuals. A–D beside the lanes for primer OPF12 identify the loci that are diagrammed in Fig. 1.

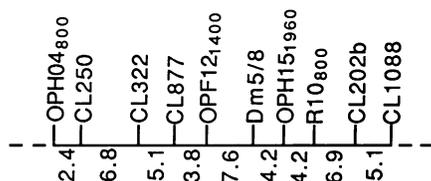


FIG. 5. Genetic map of the region of the lettuce genome containing *Dm5/8*. Three RAPD markers, *OPH04800*, *OPF121400*, and *OPH151960* were identified by bulked segregant analysis. The CL prefix designates a RFLP locus detected by a cDNA clone. Markers flanking this region have been described (5, 6). Genetic distances were derived by multipoint analysis and are shown in centimorgans. Pairwise recombination distances between the markers and *Dm5/8* are reported in the text.

using the basic mapping population. Two-point analysis showed the new markers to be 6, 8, and 12 cM from *Dm5/8*. Multipoint analysis indicated the map positions shown (Fig. 5).

## DISCUSSION

Several approaches have been suggested to saturate genomic regions of interest with molecular markers. These include preselection using NILs (4), preparative pulsed-field gel electrophoresis (10), and chromosome walking and jumping (11). Bulk segregant analysis provides a rapid, technically simple alternative for identifying markers linked to specific genes. The only prerequisite is the existence of a population resulting from a cross that segregates for the gene of interest. The success of the approach will depend on the genetic divergence between the parents in the target region.

The underlying principle of bulked segregant analysis is the grouping of the informative individuals together so that a particular genomic region can be studied against a randomized genetic background of unlinked loci. The minimum size of the bulk will be determined by the frequency with which unlinked loci might be detected as polymorphic between the bulked samples. This in turn will depend on the type of marker being screened (dominant or codominant) and the type of population used to generate the bulks ( $F_2$ , backcross, full sib, etc.). For a dominant RAPD marker segregating in an  $F_2$  population, the probability of a bulk of  $n$  individuals having a band and a second bulk of equal size not having a band will be  $2(1 - [1/4]^n)(1/4)^n$  when the locus is unlinked to the target gene. Therefore, few individuals per bulk are required. For example, the probability of an unlinked locus being polymorphic between bulks of 10 such individuals is  $2 \times 10^{-6}$ . Even when many loci are screened, the chances of detecting an unlinked locus are small. As smaller bulks are utilized, the frequency of false positives will increase. However, as the linkage of all polymorphisms is confirmed by analysis of a segregating population, bulked segregant analysis with only small numbers of individuals in one or both bulks will provide great enrichment for markers linked to target loci.

Bulked segregant analysis successfully identified markers linked to *Dm5/8* for which no NILs exist. The procedure was rapid; it required fewer than 300 PCR reactions to identify and map three new markers. The 100 primers screened  $\approx 900$  loci. Screening more primers should identify more closely linked markers, assuming a random distribution of loci detected as RAPD markers and sufficient polymorphism in the target region. The first assumption has yet to be tested, but mapping data generated in this and other labs indicate that RAPD markers are at least distributed throughout the genome. Calmar and Kordaat represent distinct types within the cultivated species; arbitrary primers detected an average of 0.53 polymorphism per primer between these two parents (R.V.K., unpublished results). Polymorphisms may be de-

tected even more frequently in the *Dm5/8* region because this gene was originally introgressed from the wild species, *L. serriola*.

The observed experimental sensitivity of bulked segregant analysis correlated well with that predicted from reconstruction experiments and studies with known markers. All polymorphic loci assayed within 15 cM of the target locus are likely to be identified; loci further away will be detected with decreasing frequency as genetic distance increases. Similar results were obtained with RFLP and RAPD markers. The narrow width of the genetic window for RAPD markers was not anticipated; in theory, the sensitivity of PCR might be expected to reveal alleles even when rarely present in the mixture. In practice, even alleles as prevalent as a proportion of 0.1 of a mixture were barely detectable. This probably reflects the competition that occurs during the initial cycles of RAPD amplification between templates with various degrees of mismatch with the primer. Precise sensitivity will vary with the sequence amplified for RAPD markers and with the age of the blot and the particular probe for RFLP markers. The width of the genetic window will also depend on the segregating population used to construct the bulks. Any segregating population originating from a single cross can be used; bulks made from backcross populations would provide greater focus around the region of interest than  $F_2$  populations, which provide maximal genetic width of the region screened for polymorphism. If sufficient individuals are pooled to form each bulk, the genetic window will be symmetrical around the target locus; this is in contrast to the region around a locus selected during the generation of NILs, which may be extremely asymmetrical (12).

Bulked segregant analysis will allow rapid mapping of loci that do not segregate in the original populations used to develop the genetic map. Bulks for the unmapped locus would be made from a new population segregating for that locus and screened using markers known to be spaced at  $\approx 30$ - to 40-cM intervals through the genome. If RFLP markers were being used, Southern blots could include multiple pairs of bulks for several loci segregating in different populations. Probes could be combined so that the whole genome could be screened rapidly to locate several loci simultaneously. Once markers that distinguish the bulks are identified, precise linkage distance could be determined by segregation analysis. If differences between a pair of bulks are not detected with existing RFLP or RAPD markers, the bulks would be screened for further markers using additional arbitrary RAPD primers; new polymorphic markers and therefore the locus would then be mapped by using an already characterized population. If only the approximate genetic position is required for a trait, the individuals segregating for the trait could be bulked prior to DNA extraction, necessitating only two extractions; the genetic position would be fixed by analyzing the ability of a series of linked markers in the region to distinguish the bulks.

Bulked segregant analysis overcomes several problems inherent in using NILs or cytogenetic stocks to identify markers linked to particular genes. There is minimal chance that regions unlinked to the target region will differ between the bulked samples of many individuals. In contrast, even after five backcrosses, only half the loci polymorphic between NILs are expected to map to the selected region (13). Linkage drag of large regions of DNA associated with the selected region in NILs (12) will not be problematic. In control experiments and in bulks from an  $F_2$  population, RFLP and RAPD polymorphisms were not detected further than 30 cM from the target locus. As bulked segregant analysis detects polymorphic loci using a segregating population, all loci detected will segregate and can be mapped. Some of the loci we have detected as polymorphic between NILs did not segregate in any of the populations we are

currently mapping (8). Near-isogenic lines require many backcrosses to develop and are therefore time consuming to generate; in contrast, bulked segregants can be made immediately for any locus or genomic region once the segregating population has been constructed. In addition, bulked segregants can substitute for cytogenetic stocks such as substitution and addition lines, for assigning probes to linkage groups or chromosome arms (14, 15), because bulks can be accurately made for particular regions as needed and do not require extensive cytological manipulations to generate and maintain.

Bulks can be made to identify markers in regions that lack markers, such as gaps in the genetic map or ends of linkage groups. To fill a gap in the genetic map, two bulks would be made from the segregating F<sub>2</sub> individuals of the mapping population. Each bulk would be homozygous for each non-recombinant genotype for the interval; recombinants would be excluded from the analysis. The bulks would be screened for RAPDs; as only two reactions are required for each primer and each primer detects 5–10 loci, hundreds of loci can be screened per day. Linkage would be confirmed by segregation analysis. Similarly, bulked segregant analysis can be made sequentially for neighboring regions to define the genetic end of a linkage group. Bulks would be made of each homozygous genotype for the terminal locus of a linkage group. If new markers are identified that are distal to the original terminal marker, bulked segregant analysis will be repeated until no more distal markers are identified and the genetic end of the linkage group is reached. This would be "genetic walking" along the chromosome: a locus identified in one round of bulked segregation analysis is used to generate the bulks for the next round. Such an analysis would consolidate the genetic map until the number of linkage groups equaled the chromosome number, unless a large region is nearly monomorphic between the two parents or there are regions of extremely frequent recombination.

Bulked segregant analysis could be extended to the analysis of genetically complex traits by screening bulks of informative individuals. If a quantitative trait is controlled by a few major genes (QTL), comparison of bulks of extreme individuals could rapidly identify markers linked to QTL. This could be made more powerful by progeny testing the extreme individuals and discarding those that do not show heritable variation. Bulked segregant analysis may also be useful in mapping loci showing partial penetrance, such as some disease loci in humans. A bulk of those progeny expressing the trait would be compared to the parents or a bulk of nonexpressing progeny (depending on the dominance relationships and the homozygosity of the parents).

Bulked segregant analysis should also be useful in analyzing species that are obligatorily outbreeding as in most animal species. In obligatorily outbreeding species, if the two parents originate from an interbreeding population, linkage

equilibrium between some loci and the target locus may prevent their detection by bulked segregant analysis. However, if individuals from only a single family are bulked, bulked segregant analysis should identify some linked markers; only when the same alleles segregate in the gametes of both parents but in linkage equilibrium with the target locus will differences not be detected between the bulks. Bulking individuals from multiple families will increase the probability that linkage equilibrium will obstruct bulked segregant analysis. The challenge for human genetics will be to identify individual families of sufficient size to allow informative bulking. Even bulks made from small families will provide great enrichment for linked polymorphic markers; linkage can be subsequently confirmed by segregation analysis of many families.

We thank S. V. Tingey, J. A. Rafalski, and J. G. K. Williams (DuPont and Nemours) for their help with RAPD markers and R. Saul (Operon Technologies) for providing many of the oligonucleotide primers. We gratefully acknowledge the support of U.S. Department of Agriculture Grant USDA-88-CRCR-37262-3522.

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