Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations

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ABSTRACT

We present a general method for isolating molecular markers specific to any region of a chromosome using existing mapping populations. Two pools of DNA from individuals homozygous for opposing alleles for a targeted chromosomal interval, defined by two or more linked RFLP markers, are constructed from members of an existing mapping population. The DNA pools are then screened for polymorphism using random oligonuclotide primers and PCR (1). Polymorphic DNA bands should represent DNA sequences within or adjacent to the selected interval. We tested this method in tomato using two genomic intervals containing genes responsible for regulating pedicle abscission (jointless) and fruit ripening (non-ripening). DNA pools containing 7 to 14 F2 individuals for each interval were screened with 200 random primers. Three polymorphic markers were thus identified, two of which were subsequently shown to be tightly linked to the selected intervals. The third marker mapped to the same chromosome (11) but 45 cM away from the selected interval. A particularly attractive attribute of this method is that a single mapping population can be used to target any interval in the genome. Although this method has been demonstrated in tomato, it should be applicable to any sexually reproducing organism for which segregating populations are being used to construct genetic linkage maps.

INTRODUCTION

RFLP mapping overcomes resolution limitations of classical genetic maps by sampling a genome at random without regard to morphological phenotype. As a result, high marker density genetic maps can be generated from molecular probes which can be subsequently utilized as tools for breeding selection, analysis of genome organization and evolution, and ultimately as starting points in genomic walking and jumping experiments (2) designed

to isolate specific loci based on map position. To date, RFLP maps have been developed for a variety of eukaryotic systems (3, 4, 5, 6, 7). Through a number of techniques, RFLP markers have been identified which are tightly linked to important genes of mammals and plant species (2, 8). In addition, recent reports have demonstrated the usefulness of tightly linked molecular markers in map-based cloning of several human disease genes (2, 9).

The most common method of placing molecular markers on a linkage map is by random cloning of genomic or cDNA sequences followed by RFLP linkage analysis. This method is extremely useful and can be used to construct both low and high resolution maps of complex genomes (2,3, 10). Nevertheless, this approach becomes limiting when one is interested in targeting a particular region in the genome. A majority of random markers will ultimately map outside of any target interval and as the interval size decreases so will the odds of any new randomly generated marker being placed within it. One solution to this problem is the use of near isogenic lines (NILs) to detect molecular markers specific to a region of interest. Young et al. (8) used NILs and pools of RFLP probes to detect new markers within the Tm-2a region of tomato. Using a similar strategy, Martin et al. (11) were able to use random polymerase chain reaction (PCR) amplification on NILs to isolate new markers near the tomato Pto disease resistance locus. Unfortunately, for most potential target intervals NILs are not available and are too time-consuming to generate. A technique to target specific chromosomal regions for marker isolation which does not require highly specialized genetic materials would be of paramount utility.

We present here a method for the rapid isolation of molecular markers specific to discrete genomic intervals defined by RFLP markers. This technique is based on utilization of a set of random PCR primers in conjunction with a population segregating for markers defining the target interval, though not necessarily segregating for any particular locus within it. Intraspecific or interspecific crosses between parents with sufficient sequence divergence to yield polymorphisms for molecular markers (for

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example those used in generating mapping populations) will result in populations amenable to the isolation of markers from virtually any interval of interest. Target intervals could include those containing specific genes, quantitative trait loci, chromosomal components such as centromeres, recombination hot spots, gaps in RFLP maps, and virtually any other genomic region of interest. Moreover, virtually any interval can be targeted using the same segregating population.

Briefly, individuals are selected from a segregating population (e.g. F2) that are homozygous across a target interval based on known markers. DNA from these individuals is combined into two pools: one homozygous for one parental type and one homozygous for the other. The result is two DNA pools homozygous at all loci within and adjacent to the target region. However, the homozygous target region differs between the two pools in parental origin, thus providing the basis for selection of polymorphic markers specific to the targeted region. A sufficiently large number of individuals in each pool assures a high probability for the presence of genomic sequences derived from both parents for all loci except those most tightly linked to and included within the target interval. Pooled DNA samples are subsequently utilized as templates for random primer amplification via PCR (1). The amplified products derived from each pool with a specific random primer are then compared by gel electrophoresis for the occurrence of polymorphism between the two pools. Because the pools are essentially homogeneous for all genomic sequences except those within and adjacent to the target interval, polymorphisms should result only if the primer primed within or adjacent to the target interval. Finally, proof of marker localization within the target interval is obtained through segregation analysis of either the polymorphic PCR product or an RFLP detected via hybridization of the purified polymorphic DNA band.

Two genomic intervals from tomato, a 15 cM interval on chromosome 10 and a 6.5 cM interval on chromosome 11, were selected to test this approach. Tomato was selected as a model system in which to test this technique because of the availability of a high resolution RFLP map (7) and a corresponding mapping population from which to generate interval specific pools. These particular regions were selected due to their lack of molecular markers and because two agriculturally important genes, which we have targeted for map-based cloning, have been tentatively localized to these regions. Specifically, the chromosome 11 interval contains the *jointless* locus (*j-1*) which regulates the development of pedicel abscission zones (12) while the chromosome 10 interval contains the *non-ripening* locus (*nor*) which regulates fruit ripening (13).

MATERIALS AND METHODS

Plant DNA isolation and pooling

DNA was isolated from F2 plants from a mapping population as previously described (7). DNA from selected plants was quantified on agarose gels and approximately 2 μg of each sample were combined. The combined mixture was diluted 200 fold in water for a final approximate concentration of 20 ng/μl.

Isogenic DNA Pool-A (IDP-A) contained DNA from the following plants which are homozygous *L. esculentum* for the genomic interval between RFLPs TG523 and CT168: 1, 5,14, 20, 48, 56, 79. IDP-B contained DNA from the following plants which are homozygous *L. pennellii* for the genomic interval between RFLPs TG523 and CT168: 19, 22, 54, 61, 76, 82, 87.

IDP-C contained DNA from the following plants which are heterozygous for the genomic interval between the RFLPs C-T16 and CT234: 8, 19, 25, 28, 31, 43, 67, 74, 80, 82, 83, 87, 90, 97. IDP-D contained DNA from the following plants which are homozygous *L. pennellii* for the genomic interval between RFLPs CT16 and CT234: 5, 9, 16, 18, 30, 32, 35, 36, 42, 45, 59, 62, 84, 88. [Note: plant numbers refer to those in Figure 3a].

RAPD amplification and analysis

The 200 random 9 and 10 base primers used were previously shown to amplify tomato genomic DNA (J. Giovannoni; unpublished results). PCR reaction conditions, final volume 25 μl, were as follows:10mM Tris-HCl pH8.3, 50mM KCl, 1.9mM MgCl₂, 100μg/ml gelatin, 0.1mM of each deoxynucleotide triphosphate, 0.5 units Cetus AmpliTaq, 1.6μg/ml template DNA, and 0.24μM primer. Each amplification reaction contained a single unique random primer. The reaction was overlayed with 20 μl of mineral oil. PCR reactions were carried out using a Perkin-Elmer-Cetus thermocycler with the following profile: 1) 94°C for 3 min×1 cycle; 2) 94°C for 1 min, 37°C for 2 min, 72°C for 2 min×45 cycles; 3) 72°C for 10 min×1 cycle. PCR reactions were analyzed by agarose gel electrophoresis using:
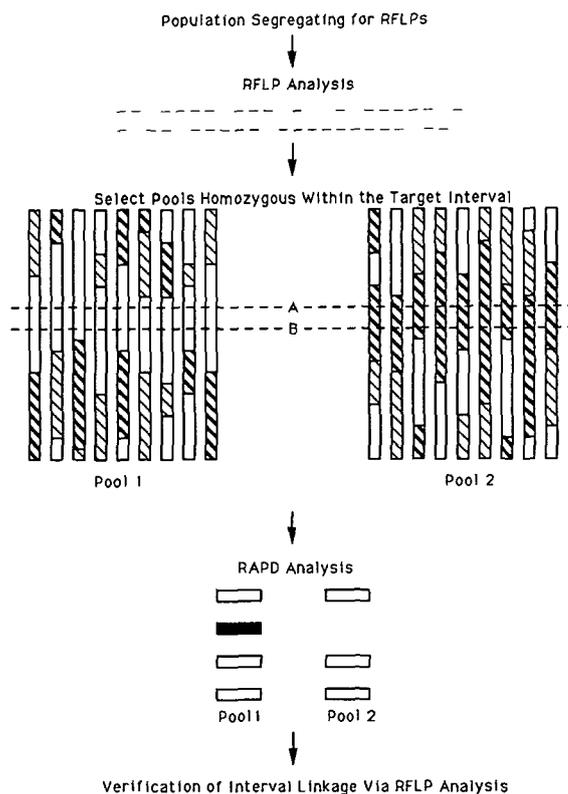
Figure 1. Schematic description of generation and use of isogenic DNA pools. Based on RFLP analysis, individual members of a segregating population are divided into two pools homozygous in the target interval for each of the parental genotypes. Bars represent net chromosomal composition of individual members of a segregating population. Open bars designate chromosomal regions homozygous for one parental genotype, lightly crosshatched bars designate heterozygous chromosomal regions, and heavily crosshatched bars designate chromosomal regions heterozygous for the other parental genotype. A and B represent two linked RFLP markers defining the borders of a target interval. Random primer amplification of isogenic DNA pools is subsequently employed to identify molecular markers specific to the targeted region.

1% agarose/1% Nusieve GTG agarose (FMC Bioproducts) in neutral electrophoresis buffer. Gels were run at 90 mA for 16 hours, stained with 0.5 µg/ml ethidium bromide and photographed. PCR reactions showing polymorphisms were repeated 1 to 3 times to control for amplification artifacts. Reproducible polymorphisms were excised from gels using a Pastuer pipet in the form of agarose plugs (approximate volume 6−8 µl) and reamplified in a volume of 100 µl as above. The resulting amplified product was gel purified using either the BioRad PREP-A-GENE kit or the freeze squeeze procedure (14).

Genetic mapping of polymorphisms

Gel purified DNA bands were radioactively labeled using the random primer method (15) and hybridized to enzyme survey filters to determine the complexity of the DNA fragment and to detect RFLPs between *L. esculentum* and *L. pennellii*. DNA fragments that represented single or low copy number sequences and displayed a RFLP were hybridized to DNA immobilized on Hybond N+ membranes (Amersham) containing appropriately digested DNA from the tomato mapping population described previously (7). Autoradiographs were scored for RFLPs and linkage analysis was performed with MAPMAKER software (16).

RESULTS

Isogenic DNA generation for specific genomic intervals

Figure 1 is a schematic representation of the generation and use of isogenic DNA pools to isolate markers between two linked RFLPs. First, an interval is selected between two mapped RFLPs. Second, plants are selected and divided into two groups which are either 1) homozygous for each parental genotype for both RFLP markers defining the target interval or 2) one pool homozygous for one parental genotype for both RFLP markers and the other pool heterozygous for RFLP markers defining the target interval. Third, DNA from the selected plants are pooled in equal proportions and the resulting mixtures are screened with random decamers in the polymerase chain reaction (1). The amplified products are then analyzed by gel electrophoresis.
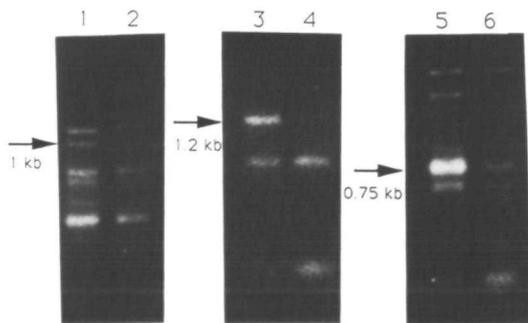

Figure 2. Electrophoresis of 3 random primer amplification reactions showing PCR polymorphisms. Ethidium bromide stained bands represent PCR amplified genomic sequences using the 3 random primers described in the legend of Figure 3B. Lanes 1 and 2; PCR amplification of isogenic DNA pools (IDPs) B and A, respectively, with primer 38D. Lanes 3 and 4; PCR amplification of IPDs B and A, respectively, with primer 148B. Lanes 5 and 6; PCR amplification of IPDs D and C, respectively, with primer 307D. Arrows designate polymorphic DNA bands. Numbers below arrows indicate molecular weight in kilobase pairs.

Reactions that show reproducible polymorphisms are then gel purified, cloned, and mapped by conventional RFLP linkage analysis.

We selected two regions of the tomato genome to test whether markers could be isolated from isogenic DNA pools. A total of 200 random unique 9 or 10 base oligonucleotides were utilized

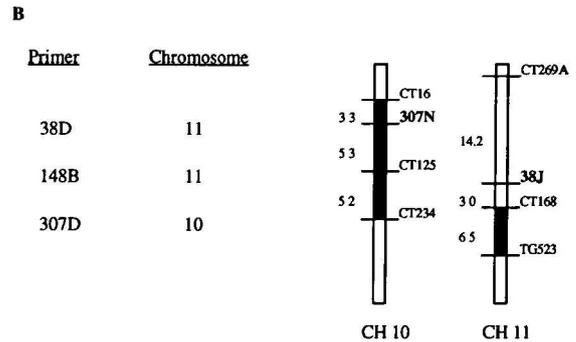
Figure 3. Linkage placement of 38J and 307N molecular markers within isogenic DNA pool intervals. A: Numerical scoring data of RFLP segregation for molecular markers defining the chromosome 10 and chromosome 11 selected intervals for those individuals comprising the isogenic DNA pools. A population of 67 F2 progeny from a *L. esculentum* X *L. pennellii* cross were screened by Southern analysis (20) to determine the allele composition of individual progeny for each RFLP marker. Those individuals homozygous across either of the two target regions, as defined by flanking RFLP markers, were selected to generate isogenic DNA pools and are listed here. The following score interpretation was employed: 1—homozygous for the *L. esculentum* allele; 2.—heterozygous; 3.—homozygous for the *L. pennellii* allele; 4—carrying the *L. pennellii* allele with inconclusive data regarding the the *L. esculentum* allele; 5.—carrying the *L. esculentum* allele with inconclusive data regarding the *L. pennellii* allele; and 0—inconclusive data regarding both the *l. esculentum* and *L. pennellii* alleles. Inconclusive data for one or both alleles usually reflects the presence of DNA bands of similar molecular weight derived from homologous loci, or technical problems with gel-blotting, hybridization, or autoradiography. RFLP marker designation is as follows: C-T269A; CT168; TG523; CT16; CT125 and CT234. Markers 38J and 307N are those isolated from the selected chromosome 10 and 11 intervals respectively, via random primer amplification of isogenic DNA pools. Plant numbers designate the individual F2 progeny in each pool. B: Chromosomal assignment of polymorphic DNA bands resulting from isogenic DNA pool amplification with random primers 38D (5'-GAAGTTGCC-3'), 148B (5'-GAAACCAGTC-3'), and 307D (5'-CTATCGGAGG-3'). Chromosomal placement of markers 38J and 307N, resulting from PCR amplification with primers 38D and 307D respectively, relative to RFLP markers used for isogenic DNA pool selection is presented diagrammatically. Blackened area designates the selected interval. Numbers between markers represent distance in cM resulting from linkage analysis performed with a subset of the *L. esculentum* X *L. pennellii* F2 population described above and MAPMAKER software (16).

in independent PCR amplifications of four pooled genomic templates designated: A) homozygous for *L. esculentum* RFLPs TG523 and CT168 defining a 6.5 cM interval harboring the *j-1* locus, B) homozygous for *L. pennellii* RFLPs TG523, and C-T168; C) heterozygous for RFLPs CT16, CT125 and CT234 defining a 15 cM interval containing or adjacent to the *nor* locus and, D) homozygous for *L. pennellii* RFLPs CT16, CT125 and CT234. Pool C was constructed from individuals heterozygous for the target interval because sufficient plants homozygous for *L. esculentum* RFLPs in this region were not available from the mapping population. The pitfall of heterozygous interval usage is that target region alleles from only one parent differ between the two pools. Thus, twice the number of random primers must be screened to yield the same probability of identifying a polymorphic marker as with pools homozygous for the target interval.

Seven to 14 individual plants were used to generate each pool (see discussion for optimal pool sizes). All reactions resulting in putative polymorphisms were repeated one to three times to minimize amplification artifacts. A total of three putative interval linked markers were identified using these criteria (Figure 2) and subsequently tested for linkage to target intervals by RFLP segregation analysis. Two markers were specific to pools A and B (*j-1* locus) and one was specific to pool D (*nor* locus). RFLP linkage analysis showed that polymorphic bands 38J and 307N resulting from primers 38D and 307D showed tight linkage to the *j-1* and putative *nor* intervals, respectively. Figure 3 depicts the mapping data numerically and indicates placement of the new markers relative to their respective intervals. The putative *j-1* linked amplification product derived from primer 148B mapped to a region of chromosome 11 approximately 45 cM away from the interval targeted with pools A and B. Examination of segregation data for the 148B derived marker region indicate that pools A and B were skewed such that each received greater than 75% of their respective DNA sequence from alternate parents (Figure 3a). Thus, the polymorphism resulting from amplification with primer 148B may reflect competition between priming sites in an inadvertently skewed region. One would anticipate that increasing the number of individuals comprising a given set of pools would decrease the potential for skewing in any particular region. To test this hypothesis, 2 pools of 15 F2 progeny derived from another mapping population (R. Wing, unpublished), and

homozygous for each parent within the chromosome 11 interval, were tested for the presence of the 148B amplification polymorphism and compared to the original 7 plant pools A and B. Increased pool size resulted in considerable reduction in the intensity of the polymorphism detected with primer 148B, although it was not eliminated completely (data not shown). We conclude that inadvertent skewing, in combination with differential competition for priming, may result in false positives. In addition, increased pool size may reduce the frequency of this phenomena.

DISCUSSION

Isolation of markers linked to intervals

We have demonstrated a technique for the isolation of molecular markers specific to discrete genomic intervals through the use of isogenic DNA pools. A total of 4 genomic pools representing 2 tomato genomic intervals defined by RFLPs, and their respective controls, were screened for PCR amplification polymorphisms with random oligonucleotides. One pool isogenic for a 6.5 cM interval on tomato chromosome 11 (Figure 3b) was screened with 100 primers and yielded two reproducible polymorphisms. Both amplification products mapped to chromosome 11. One (38J) resided adjacent to the selected interval, while the other (148J), mapped approximately 45 cM from the interval border. One would expect to isolate markers linked to but outside the selected interval due to linkage drag (17). However, the great genetic distance between the chromosome 11 interval and 148J (45 cM) suggests that this particular polymorphism may have resulted from unintentional skewing of another genomic interval, in concert with competition for priming sites which exist in different ratios between the two DNA pools (18; J. Giovannoni unpublished results). Marker 38J mapped 3 cM adjacent to the defined target interval and fortuitously narrowed a 17 cM gap between RFLPs CT168 and CT269A on the tomato map (Figure 3b.).

In addition, two DNA pools homozygous and heterozygous for a 15 cM interval on chromosome 10 were screened with 200 random primers. One amplification polymorphism resulted (307N) which mapped to the selected interval (Figure 3b). The chromosome 10 pools differed from those used with chromosome 11 in that one of the chromosome 10 pools was heterozygous for RFLP markers spanning the interval while the other chromosome 10 pool and both chromosome 11 pools were homozygous for markers across their respective intervals (as in the model situation depicted in Figure 1). Use of a heterozygous interval pool should result in a 50% decrease in the frequency of polymorphism detection since target interval DNA representative of only one of the population parents (and thus any corresponding polymorphic sequences) are unique to only the heterozygous interval pool. As a consequence, comparison of PCR reactions between pools homozygous and heterozygous across the selected interval would be expected to yield additional amplified sequences only with the heterozygous DNA template. Interestingly, marker 307N was originally detected as a polymorphism specific to the homozygous interval DNA pool (data not shown). Subsequent amplification reactions demonstrated the presence of the 307N amplification product in both chromosome 10 pools although with much greater intensity in the homozygous interval DNA pool (Figure 2). This phenomenon may be due to competition for available primers by sequences found only in the heterozygous interval pool. Alternatively, the
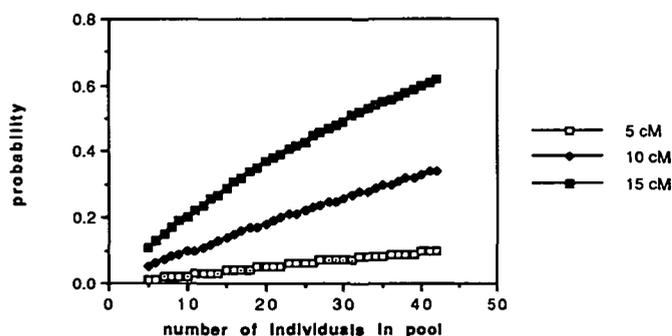

Figure 4. Double crossover probability. Plot of the number of individuals in a pool versus the probability of at least one individual containing a double crossover within the targeted interval. Calculations are for 5 cM, 10 cM, and 15 cM intervals and based on selection of F2 individuals homozygous for markers flanking the targeted interval.

polymorphism may be due to the reduced level of 307N template in the heterozygous isogenic DNA pool-C. Nevertheless, this result demonstrates both the infidelity of random primer PCR amplification of pooled genomic sequences and the potential benefits of further examination of polymorphisms showing reproducible differences in band intensity between isogenic DNA pools.

The use of a homozygous and heterozygous pool is analogous to pools that would result from a testcross population. In fact, any population segregating for a specific target interval can be used to generate isogenic DNA pools for interval linked marker isolation (e.g. F2, backcross, recombinant inbreds).

Intervals versus points

Michelmore et al. (18) has recently reported the successful use of random primer amplification of pooled ('bulked') DNA samples to isolate 3 molecular markers linked to the lettuce downy mildew resistance gene Dm5/8. Although the method described herein shares technical similarity in the use of random primer amplification of pooled DNA samples, several significant differences in methodology and potential application are apparent. Michelmore et al. generated two pooled DNA samples homozygous for either of the alternate resistant or susceptible Dm5/8 alleles. Consequently, selection of pool members was based on homogeneity at a single genetic point in a population segregating for the target of interest.

The selection and use of DNA pools isogenic for genetic intervals (described here) versus single points differ in at least four important aspects: 1) For the applications described here, genetic loci which do not segregate in the population used for isogenic DNA pool generation can still be targeted if flanking RFLP markers have been identified; 2) chromosomal structural and functional loci, such as centromeres, which cannot be scored directly in segregating populations, can still be targeted if an interval in which they reside has been generally defined; 3) gaps in genetic maps can be more efficiently filled via selection for both markers bordering the interval as opposed to just one. A combination of selection for pool members homozygous for two or more RFLP markers defining a target interval but heterozygous for flanking RFLP markers can be utilized to increase the likelihood that any polymorphic amplification product will be derived from sequences internal as opposed to adjacent to it. 4) DNA samples from the same segregating population can be pooled in different combinations to target any interval in the genome. For many plant and animal species mapping populations already exist and are the basis of existing RFLP maps (19). These populations can be put to use immediately to target markers to gaps in existing maps or to isolate markers in intervals likely to contain genes of interest.

Optimum interval and pool sizes

A description of the interval pooling technique would not be complete without a discussion of the optimal pool and interval sizes. Pooling larger numbers of individuals increases the probability that the two pools will not differ for alleles other than those within and adjacent to the target interval. However, as the pool size increases, so does the probability that individuals will occur in the pool with a double crossover within the interval. If such individuals do occur in a pool, they could reduce or eliminate the chances of finding a PCR-derived marker in the region affected by the double crossover. This is especially of concern when the intervals are large and selected only by genetic markers flanking the interval. If additional information is available about genetic markers within the interval, the probability of double crossover individuals decreases.

Figure 4 is a plot of the probability of at least one double crossover occurring in a pool of n F2 individuals. For a 5 cM interval the probability is less than 0.10 (10%) for pools of 40 or less. As the targeted interval increases to 10 cM, fewer than 10 pooled individuals can be pooled to maintain the same probability of double crossovers. A 15 cM interval would allow pooling of 5 or less individuals. However, with decreasing numbers of samples, there is an increasingly significant chance that the pools will share areas of homozygosity other than the targeted interval, thus resulting in increased numbers of false positives during the PCR screening. Consequently, we recommend that the pools should contain greater than 5 individuals. Five F2 individuals per pool results in a probability of 0.97 (97%) that any non-targeted genomic region will be represented by both parents. We also estimate that the genetic distance of targeted intervals should be less than 15 cM. The two intervals targeted here were 6.5 and 15 cM with pool sizes of 7 and 14 individuals, respectively. The 15 cM chromosome 10 interval contains an internal RFLP marker, CT125, which was also scored for pool selection (Figure 3a), effectively making this interval two adjacent 6 and 9 cM intervals.

SUMMARY

The method described in this paper provides a rapid and efficient means for the isolation of molecular markers linked to any defined genomic interval. This is especially useful when there are no other markers available within the region of interest. The fact that this technique is based on PCR and utilizes existing mapping populations should make it applicable to both plant and animal systems. Prerequisite materials for implementation of this procedure include a population segregating for the genomic interval of interest, a set of random primers, and a DNA thermocycler. The segregating population serves both as substrate for interval-specific pool generation and as a mapping population for linkage verification of putative markers.

We are currently constructing and screening isogenic DNA pools, using the same F2 population, for intervals where map-based cloning target loci have been mapped and where gaps exist in the tomato RFLP map. This technique can be used to target any important locus including structural and regulatory genes, chromosomal components such as centromeres, and will also provide markers for map-based cloning and breeding programs. Overall, this technique may ultimately prove useful for the isolation of markers in a wide range of systems.

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