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Utility of RAPD markers in identifying genetic linkages to genes of economic interest in peach

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Abstract The identification of molecular markers linked to economically important traits for use in crop improvement is very important in long-lived perennial species. Three-hundred-and-sixty RAPD primers were used with bulked segregant analysis to identify markers linked to loci of specific interest in peach [(*Prunus persica*) L. Batch] and peach × almond [(*Prunus dulcis*) Batch] crosses. The traits analyzed included flesh color, adhesion, and texture; pollen fertility; plant stature; and three isozyme loci. The Mendelian behavior of the RAPD loci was established, and RAPD markers were mapped relative to the loci controlling flesh color, adhesion, and texture, and the isozyme loci *Mdh-1*, *6Pgd-2* and Aat-1, as well as the existing RFLP genetic linkage map constructed previously using a peach × almond F_2 population. This technique has facilitated rapid identification of RAPD and RFLP markers that are linked to the traits under study. Loci controlling these traits mapped predominantly to linkage groups 2 and 3 of the peach genetic linkage map. Linkages to genes with both dominant and co-dominant alleles were identified, but linkages to dominant genes were more difficult to find. In several crosses, RAPD marker bands proved to be allelic. One co-dominant RAPD formed a heteroduplex band in heterozygous individuals and in mixtures of alternate homozygotes. The Mendelian behavior of the RAPD loci studied was established and the results suggest that RAPD markers will be useful for plant improvement in peach.

Key words RAPDs · *Prunus* spp. · Bulked segregant analysis (BSA) · Co-dominance · Heteroduplex

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Introduction

Peaches [*Prunus persica* (L.) Batch] are grown extensively throughout the U.S. and both peaches and almonds (*Prunus dulcis* Batch) are crops of major economic importance in California. Due to the long juvenile period and large plant size, genetic improvement of fruit tree crops has been difficult and time consuming. Many of the cultivars currently planted in commercial peach and almond orchards in the U.S. were developed over 100 years ago.

Tree breeding will benefit from the use of molecular markers associated with genes for horticultural traits through marker-aided selection (MAS). MAS would also allow screening for economically important traits in seedlings, which is especially useful for traits expressed only in fully mature trees. Additionally, MAS could expedite difficult screening procedures such as testing for disease or insect resistance or searching for genes that may express only partial penetrance or expressivity (Haley et al. 1993). Further benefits of well-characterized molecular markers include the ability to screen large germplasm collections for either specific traits or for additional genetic diversity.

Genetic studies in peach, while more advanced than other *Prunus* species, lag behind many other crops (Hesse 1975). Until very recently, little segregation data and essentially no genetic linkage data were available on morphological or biochemical traits. Recent mapping studies using molecular markers have provided genetic linkage maps of the peach genome, although to-date, few morphological traits have been mapped (Chaparro et al. 1994; Foolad et al. 1995; Rajapakse et al. 1995). Identification of RAPD markers linked to traits of interest using bulked segregant analysis (BSA) (Michlemore et al. 1990) has proven to be highly efficient (Haley et al. 1993; Maisonneuve et al. 1994). We propose to use BSA and RAPDs to identify markers linked to useful genes in *Prunus* spp. and to add these markers to an existing linkage map previously constructed in this lab by Foolad et al. (1995). Adding loci governing morphological traits to the map will facilitate the study of the organization of these genes in the genome

and may lead to the isolation of genes through the use of map-based cloning. Additionally, these RAPD markers may be converted into highly repeatable and useful diagnostic markers such as RFLPs or sequence characterized amplified region (SCARs). Furthermore, placing the loci on an existing RFLP-based linkage map will identify other RFLP markers linked to these loci for future use in plant breeding.

RAPD markers usually show dominant expression and are scored for the presence or absence of each amplified band. However, co-dominant expression of RAPD bands would be expected if an inserted or deleted sequence was found within the amplified region of some, but not all, of the individuals scored for each primer. Bands of different sizes, but with identical priming sites, would characterize these allelic pairs and, like RFLPs, are expected to be very similar in DNA sequence for all except the inserted or deleted section of the amplified region. Heteroduplex formation in heterozygous individuals is a possibility during the polymerase chain reaction (PCR), although it has been rarely reported in the literature (Ayliffe 1994). Co-dominant RAPD markers would prove favorable to crop improvement via molecular markers.

Materials and methods

Segregating populations

Three segregating populations, two from peach × almond crosses and one from a peach × peach cross, were studied. PMP1 is an interspecific F_2 with 64 individuals resulting from a cross between the dwarf peach breeding line 54P455 and the almond cultivar Padre; PMP6 is a segregating F_3 population consisting of 113 individuals which resulted from selfing a single F_2 from the cross 54P455 peach × 6A-11 almond, and PMP3 is a segregating F_1 population of 112 individuals from a cross between two diverse peach breeding lines (B8-23-16 × A104-115) made by Dr. J C. Goffreda at Rutgers University. The parents of the latter population were heterozygous at many loci, and the F_1 population was found to segregate 1:1 and 3:1 for many of the RAPD bands scored (data not shown).

Traits evaluated

Individuals within each population were scored for useful traits in peach (from Hesse 1975), including white (*Y-*) vs yellow (*yy*) flesh, freestone (*F-*) vs clingstone (*ff*), normal (*St-*) vs stony hard (*stst*) flesh, tall (*Dw-*) vs brachytic dwarf (*dwdw*) stature, pollen fertility (*Ps-*) vs pollen sterility (*psps*), and *Mdh-1* fast (*FF*) vs slow (*SS*) alleles, an isozyme implicated in plant vigor by Werner and Moxley (1991). These qualitative traits are easily scored for alternate phenotypes, and show little environmental effects. The fast vs slow alleles of each *6Pgd-2* and *Aat-1* were studied first to test the suitability of BSA to this type of analysis in peach. Chi-square analyses were performed on segregating populations for each trait to verify Mendelian segregation.

DNA extraction and quantification

DNA was extracted from 5 g of fresh, washed leaves of parent and progeny trees according to Doyle and Doyle (1990), except that an SDS-Tris-EDTA extraction buffer was used instead of a CTAB buffer and the final precipitation in ethanol was eliminated. The youngest fully expanded leaves were collected early in the growing season for DNA extraction. DNA samples were digested with RNase and stored in Tris-EDTA at 4°C until ready for use. DNA was quantified either with a fluorimeter or through a comparison of DNA brightness to a standard (lambda cut with *Hin*dIII) using the IS-1000 Digital Imaging System from Alpha Innotech Corporation (San Leandro, California).

RAPD amplification

RAPD amplification was performed on 5 μl of a solution containing 20 μl/ml of DNA according to Yu and Pauls (1992) in a Perkin-Elmer-Cetus thermal cycler. Optimization of DNA concentration was performed prior to analysis to determine which concentration (20 μl/ml) provided the best amplification for the majority of the primers tested. Primers for amplification were obtained from Operon Technologies and the University of British Columbia. Amplified DNA fragments were separated on 2% agarose gels using TAE buffer and visualized with Ethidium Bromide and UV illumination. RAPDs were named according to the designation of the primer that was provided by the primer source and the molecular weight of the band (i.e., $OPO\text{-}19_{1599}$).

Bulked segregant analysis

Bulked segregant analysis (BSA) was performed by adding equal amounts of DNA from 8 to 12 individuals per bulk according to Michelmore et al. (1991). Two bulks were constructed for each trait, each representing the alternate phenotypic states of the loci controlling the selected trait. Three-hundred-and-sixty RAPD primers were amplified for each alternate bulk, and only bright, repeatable bands that were polymorphic between bulks were chosen and scored from all progeny to determine the map distance between the RAPD loci and the selected trait. Repeatability between amplifications was tested and found to be very high when all conditions are held constant (Warburton and Bliss 1996).

Test of co-dominance

Bands amplified by the same RAPD primer, and suspected to be allelic based on Mendelian segregation in the mapping populations, were tested for co-dominance using Southern hybridization. One band from each of the six possible co-dominant loci was cut from an agarose gel and the DNA extracted using the GeneClean Kit (Bio 101, Inc.). This DNA was labeled using the enhanced chemiluminescent labeling system ECL of Amersham, and used to probe a Southern blot containing all possible allelic bands. In one case, to test for the formation of a heteroduplex band, equal amounts of DNA from two alternate homozygotes were mixed and a RAPD reaction run on the mixture. The resulting amplified products were subjected to electrophoresis as described above.

Mapping traits

The population used to construct the linkage map by Foolad et al. (1995) is being maintained in an orchard at the University of California at Davis. This interspecific F_2 population, (PMP1), was used to add RAPD loci identified in this study to the map and will continue to expand the map as linkage studies are extended. Traits that do not segregate in PMP1 can be added to the map by identifying linked molecular markers in other segregating populations and then mapping these markers in PMP1. Following BSA, Linkage-1 (Suiter et al. 1986) was used to calculate the map distance between pairs of loci, and JoinMap (Stam 1993) was used to calculate the best linear order and map distance for larger linkage groups. Data from more than one population can also be combined into one linkage group using JoinMap if at least two of the loci are segregating per linkage group in both populations. The program MAPMAKER

Table 1 Monogenic segregation and chi-square values of morphological, isozyme, and RAPD[a] loci mapped in this study

Loci	Exp. ratio	Obs. ratio	χ^2	*P*	Pop.[b]
Aat-1	1:2:1	16*FF*:53*FS*:36*SS*	7.63	0.02	PMP6
BC-134:$_{2522}$	1:2:1	30*AA*:42*AB*:20*BB*	2.87	0.24	PMP3
Freestone, *F*	3 1	80*FF*-:26*ff*	0.01	0.91	PMP3
Mdh-1	1:2:1	28*FF*:51*FS*:27*SS*	0.17	0.92	PMP6
OPD-3:$_{2460}$	1:1	40*Aa*:51*aa*	1.33	0.25	PMP3
OPD-3:$_{2706}$	1:1	52*Aa*:39*aa*	1.86	0.19	PMP3
OPD-5:$_{800}$	1:2.1	25*AA*:42*AB*:22*BB*	0.48	0.79	PMP3
OPD-7:$_{627}$	3:1	60*A*-:30*aa*	3.33	0.07	PMP6
OPD-7:$_{1046}$	3:1	71*A*-:14*aa*	3.30	0.07	PMP6
OPE-7:$_{861}$	3:1	68*A*-:23*aa*	0.00	0.98	PMP3
OPF-5:$_{677}$	3:1	81*A*-:23*aa*	0.46	0.50	PMP3
OPF-5:$_{2460}$	1:1	43*Aa*:60*aa*	2.81	0.09	PMP3
OPF-5:$_{2460}$	3:1	34*A*-:10*aa*	0.12	0.71	PMP6
OPF-13:$_{1169}$	3:1	73*A*-:13*aa*	4.48	0.03	PMP6
OPF-16:$_{873}$	3:1	65*A*-:20*aa*	0.10	0.77	PMP3
OPG-16:$_{1095}$	1:1	40*Aa*:43*aa*	0.11	0.74	PMP3
OPI-7:$_{1058}$	3:1	84*A*-:16*aa*	4.32	0.04	PMP3
OPO-4:$_{923}$	3:1	52*A*-:15*aa*	0 24	0.63	PMP6
OPO-19:$_{1784}$	1:2:1	28*AA*:42*AB*:34*BB*	4.54	0.11	PMP3
OPO-19:$_{1599}$	3:1	51*A*-:22*aa*	0.39	0.55	PMP6
Stony Hard, *Sh*	3:1	75*Sh*-:30*shsh*	0.71	0.40	PMP3
Yellow, *Y*	3:1	73*Y*-:35*yy*	2 45	0.12	PMP3
6Pgd-2	1:2:1	32*FF*:56*FS*:18*SS*	4.04	0 13	PMP6

[a] RAPD loci are named using the initials of the source (OP = Operon Technologies, BC = the University of British Columbia) followed by the letter and/or number of the primer kit, followed by the size of the band amplified in base pairs

[b] Population in which the loci of interest are segregating. PMP3 = peach×peach (B8-23-16×A104-155) segregating F_1 population containing 112 individuals, and PM6 = peach×almond (54P455×6A-11) segregating F_3 population containing 113 individuals

(Lander et al. 1987) was used to determine linkage group order and the distances between markers and to add new markers to existing linkage groups.

Results and discussion

Genetic mapping

Morphological, isozyme, and RAPD loci scored in this study were shown to segregate in a Mendelian fashion in two populations, PMP3 and PMP6, prior to further use (Table 1). Linkages between RAPD markers and qualitative trait loci in *Prunus* were identified using BSA. The RAPD marker OPI-7_{1058} segregated with the freestone (*F*-) locus (Fig. 1). Other RAPDs linked to the co-dominantly expressed isozyme loci *6Pgd-2*, *Aat-1* and *Mdh-1*, and to the dominant loci for stony hard, yellow, and freestone flesh, were also identified (Fig. 2). At least two RAPD markers linked to the loci controlling *6Pgd-2*, *Aat-1*, *Mdh-1* and yellow flesh (*Y*) segregated in each population and in the mapping population PMP1. This allowed direct comparison of the linear order within specific linkage groups in multiple populations, and the addition of the RAPD markers and linked traits to the initial linkage map previously constructed using RFLPs in PMP1 (Fig. 3). The *6Pgd-2* and *Aat-1* loci mapped to linkage group 2 and *Mdh-1* and *Y* mapped to linkage group 3. These numbers represent linkage group designations followed by convention in labs

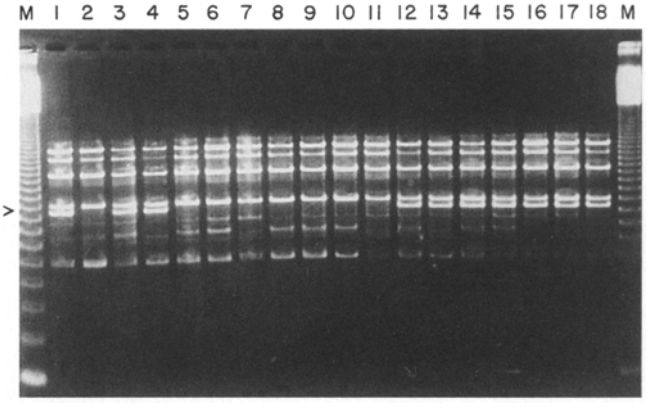

Fig. 1 Banding pattern of a RAPD marker (OPI-7_{1058}), which was found by bulked segregant analysis to be linked to the freestone locus (*F*) in PMP3, a segregating F_1 population. *Lanes 1 and 2* are the freestone and clingstone bulks, respectively, showing the polymorphism at the linked marker (*arrow*). *Lanes 3 and 4* are the peach parents B8-23-16 and A104-155, respectively. *Lanes 5–11* are clingstone progeny, *lanes 12–18* are freestone progeny, and *M* is the 123-bp molecular-size marker

working on mapping in *Prunus* and do not correspond to chromosomes because little physical mapping has been done in peach, including aligning the physical and genetic maps. However, the approximate map location of each of these genes and potentially useful RFLP markers linked to the loci governing these traits are provided. These results

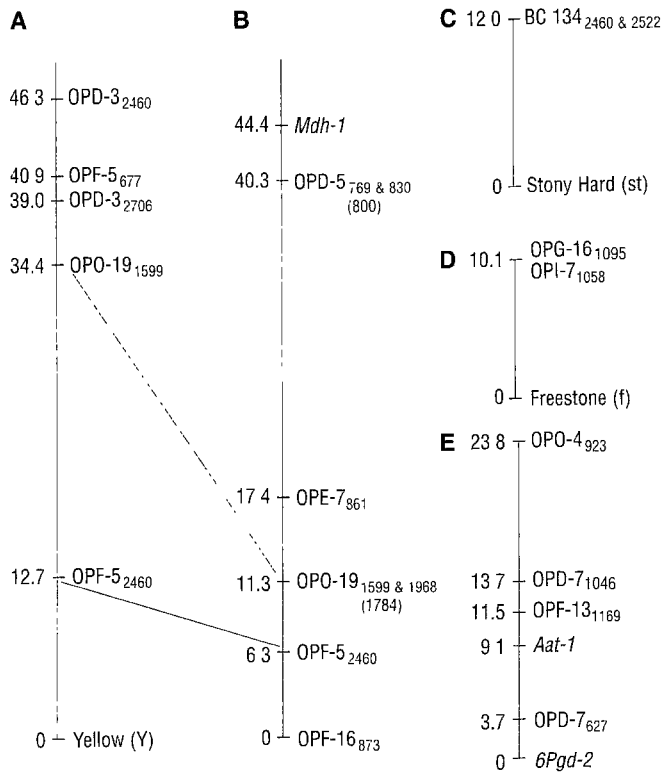

Fig. 2 Map of linkage groups identified in PMP3 and PMP6 using bulked segregant analysis to identify linkages between RAPD markers and the expressed loci yellow (*A*), *Mdh-1* (*B*), Stony Hard (*C*), Freestone (*D*), and *Aat-1* and *6Pgd-2* (*E*). Loci and populations are as described in Table 1 and distances to the left of each linkage group are expressed in cM and were calculated with the program JoinMap. *Lines* between linkage groups A and B indicate the relationship between the linkage groups calculated inPMP3 (*A*) and PMP6 (*B*), in which two common markers (OPO-19_{1784} and OPF-5_{2460}) were found to be segregating

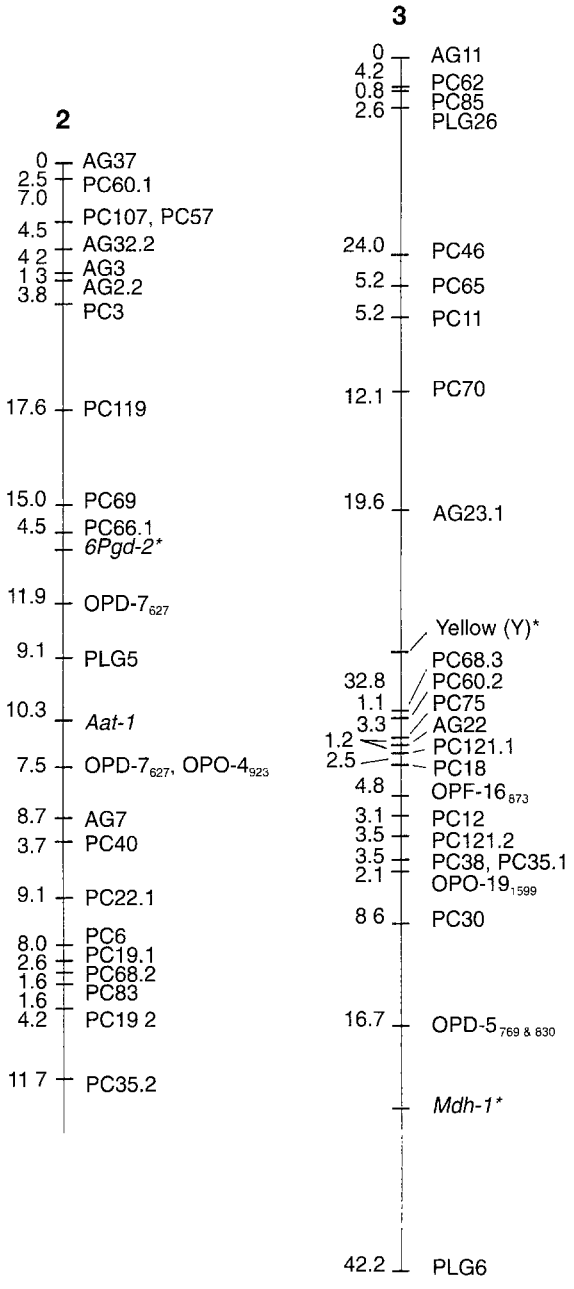

Fig. 3 Integrated map of RFLP and RAPD markers, and three isozyme loci and one morphological locus in PMP1. Names of loci are to the right of each linkage group and the genetic distances in cM are shown on the left. Loci beginning with *OP* are RAPDs; loci beginning with an *A* or a *P* are RFLP markers mapped in a previous study (Foolad et al. 1995). * = Approximate map positions based on data from PMP6. Linkage group 3 contains the linkage groups *A* and *B* reported in Fig. 2; linkage group 2 contains group *E*; Groups *C* and *D* from Fig. 2 were unable to be mapped in PMP1 because neither the morphological nor the RAPD loci segregated in that population

demonstrated the utility of BSA for adding loci of interest to a genetic linkage map even though these loci do not segregate in the original mapping population.

Limitations of bulked segregant analysis

BSA is less efficient for identifying linkages to loci with dominant rather than co-dominant alleles, because recessive alleles are present in both the recessive and dominant bulked DNA samples for the dominant case. Consequently, only linkages to the dominant alleles (linked in coupling phase) will be identified under most circumstances. Despite having screened 360 primers, no linkages were found either to the dwarf locus (*dw1*) or the pollen-sterility locus (*ps*). Although an analysis of white vs yellow bulks revealed no linkages using BSA, a RAPD marker linked to the recessive "*y*" allele (yellow flesh) was identified in another study which did not use BSA (Warburton and Bliss 1996). This linkage was used to identify probable "*Yy*" heterozygotes, which were white-fleshed individuals but contained the RAPD marker whereas homozygous white-fleshed (*YY*) individuals did not have the band (except in the case of recombination), and to exclude the heterozygotes from the white-fleshed bulk. This allowed the identification of three other RAPD loci linked to the *y* locus when these bulks were re-screened using BSA.

When screening primers against the two contrasting bulks for each trait, polymorphic bands were often ob-

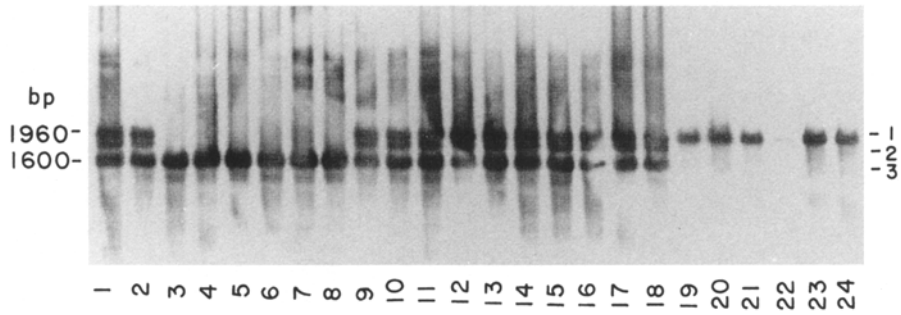

Fig. 4 Southern blot of DNA from PMP3 showing allelic segregation of fragments amplified by primer OPO-19. *Lane 1* = parent B8-23-16, *lane 2* = parent A104-115, *lanes 3–8* are F_1 individuals homozygous for the fast band *3* (OPO-19_{1599}), *lanes 9–18* are heterozygous F_1 individuals displaying the heteromorphic band *2* (OPO-19_{1845}), as well as the two parental bands, and *lanes 19–24* are F1 individuals homozygous for the slow band *1* (OPO-19_{1968}). DNA immobilized on the membrane was amplified originally with OPO-19, and the membrane was probed with labeled DNA from band OPO-19_{1599} which was obtained following a separate RAPD reaction with OPO-19

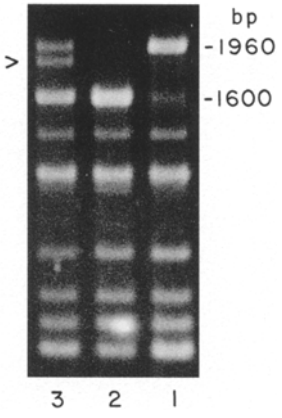

Fig. 5 Formation of a heteroduplex fragment in peach during PCR when template DNA from alternate allelic homozygotes was mixed in the same reaction tube. Equal amounts of DNA from the 1599-bp homozygote (*lane 2*) and the 1968-homozygote (*lane 1*) were mixed in a PCR reaction using the same primer (OPO-19) which produced a third band which migrated to an intermediate position on a 2% agarose gel (*lane 3*, *arrow*). The individual in *lane 1* is homozygous for the slow allele of this locus; the faint band which appears to be the alternate, fast allele is in fact a non-homologous band which comigrates to the same position. Southern hybridization has shown that it is not allelic to this locus (data not shown)

served but ultimately could not be confirmed as being linked to the trait of interest. These "false positives" occurred at a frequency of about 30%, and could be identified as non-linkages by running RAPD assays on several of the individuals with each phenotype of a trait. In a false-positive situation, the band of interest was often amplified from individuals with both phenotypes, but is only expected to amplify from the individuals with either one of the two phenotypes but not the other. The possible reasons for the false positives may include sampling error, loose linkage, or PCR competition in bulks. In a bulk, a band present at low frequency may not amplify since competition for primer sites from the many possible primer sites may be too intense (Newbury and Lloyd Ford 1993). However, some bands present at very low frequencies (i.e., present in only one individual in a bulk of ten) may successfully amplify in the bulk; this is more common with small (i.e., less than 600 bp), rather than large, bands (data not shown). Thus, any segregating RAPD band may cause a false positive if too few individuals make up the bulk. Too many individuals per bulk may also present a problem since the chances of adding a recombinant individual to the bulk increases with increasing numbers, and it may become more difficult to identify all but the tightest linkages since one or two recombinant individuals may be found in each bulk. This would present an even greater problem if RFLPs are used, since, unlike the case with competitive RAPDs, even one recombinant per bulk would negate the possibility of identifying a linkage to an RFLP. While closer linkages are desirable, this greatly reduces the efficiency of BSA in identifying linkages.

Behavior of RAPDs

Six RAPD primers each amplified two bands of different size which were found to be linked to the same gene. When map distances were calculated, three of these primers (OPO-19, OPD-5, and BC-134) had fragments that mapped to the same location and three primers had fragments that did not (OPD-3, OPF-5, and OPD-7). When scored as codominant bands, OPO-19, OPD-5 and BC-134 segregated in a ratio of 1 (small band):2 (both bands):1 (large band) (Table 1). To confirm whether or not these bands were allelic, one band of each primer was excised from an agarose gel and hybridized to the other band through Southern hybridization. This test confirmed the allelism of bands from primers OPO-19 (Fig. 4), OPD-5 and BC-134 (data not shown), but not the other three. Furthermore, in amplifications of the heterozygote and of a mixture of the two different homozygotes, OPO-19_{1968} and OPO-19_{1599} formed a heteroduplex band which migrated to an intermediate position between the two other bands in the gel (Fig. 5). This behavior was not observed for OPD-5 or BC-134.

Linkages between RAPD markers and qualitative loci were found to be conserved in more than one population. *Mdh-1* and the *Y* locus have been reported to be linked

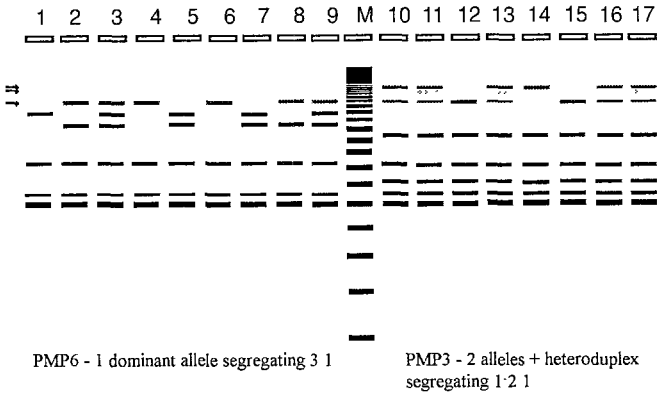

Fig. 6 Diagram of an agarose gel demostrating the co-dominant segregation of alleles at the OPO-19 locus (alleles marked by *arrows*), in the population PMP3, resulting from hybridization of two heterozygous peach parents (B8-23-16 and A104-115), and dominant behavior in the population PMP6, resulting from hybridization of a homozygous peach parent (54P455) and an almond parent (6A-11). The almond parent did not amplify a fragment at this locus and therefore did not contribute an observable RAPD band. *Lanes 1–17* are labeled as follows: *1* = almond 6A-11, *2* = peach 54P455, *3–9* = segregating F3 progeny, *10* = peach B8-23-16, *11* = peach A104-115, *12–17* = segregating F1 progeny. M = 123-bp ladder marker

(Chaparro et al. 1994). Two RAPD loci, OPO-19_{1784}/OPO-19_{1599} and OPD-5_{800}, were determined to be linked to the *Y* locus in population PMP3 and to the *Mdh-1* locus in population PMP6. Our data confirmed this same linkage group in two different populations (Fig. 2). Observation of the segregation of loci in different populations revealed the expected Mendelian behavior of these RAPDs. The alleles at the OPO-19 locus were co-dominant in PMP3 where each parent contributed a different sized band to the progeny but, when it was amplified in PMP6, the almond parent did not express a band and a 3:1 segregation ratio (presence vs absence) was observed in the F_3 population (Fig. 6).

Because of the co-dominant segregation of some RAPD fragments, these markers should be more useful in the genetic mapping of loci and marker-aided selection than previously thought. The ability to distinguish heterozygotes from dominant homozygotes greatly increases the information from each individual and thus requires fewer individuals when calculating the recombination frequency between two loci. Furthermore, MAS of dominant traits linked to dominant RAPD markers will be less efficient. When linked in coupling phase, these markers will identify individuals with at least one copy of the desired trait but will not distinguish heterozygotes; and when linked in repulsion phase, these markers will not allow the distinction of favorable genotypes (recessives) from unfavorable genotypes (heterozygotes). Co-dominant RAPDs would allow all linkage configurations to be utilized most efficiently for MAS. However, the rate at which co-dominant markers were identified in this study (3 out of 17 or less than 20%), and the even lower rate of co-dominance reported by Ayliffe et al. (1994), indicates that unless detection can be enhanced (Davis et al. 1995) these markers may be too rare to justify their use in mapping and breeding programs.

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