

A peach linkage map integrating RFLPs, SSRs, RAPDs, and morphological markers

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Abstract: A linkage map was obtained using a BC₁ progeny (*Prunus persica* × (*P. persica* × *P. ferganensis*)). The map is composed of 109 loci (74 RFLPs, 17 SSRs, 16 RAPDs, and two morphological traits) distributed in 10 linkage groups. Loci, segregating in five different ratios, were integrated in the map with JoinMap 2.0 software. The map covers 521 cM of the peach genome. The average distance between adjacent loci is 4.8 cM. Two monogenic traits, flesh adhesion (*F/f*) and leaf glands (*E/e*), were placed on the map. Thirty-two loci in common with a saturated linkage map of *Prunus* allowed a comparative analysis to be made between the two maps. Homologies were found among the respective linkage groups. No relevant differences were observed in the linear order of the common loci.

Key words: peach, linkage map, *Prunus persica*, *Prunus ferganensis*, molecular markers.

Résumé : Une carte de liaison génétique a été développée à partir d'une population BC₁ (*Prunus persica* × (*P. persica* × *P. ferganensis*)). La carte comprend 109 locus (74 RFLP, 17 microsatellites, 16 RAPD et deux marqueurs morphologiques) formant 10 groupes de liaison. Les locus, montrant un total de cinq ratios de ségrégation différents, ont été intégrés à l'aide du logiciel JoinMap 2.0. La carte couvre 521 cM du génome du pêcher. La distance moyenne entre les marqueurs est de 4,8 cM. Deux caractères monogéniques, l'adhérence de la chair (*F/f*) et les glandes foliaires (*E/e*), ont été situés sur la carte. Trente-deux locus en commun avec une carte génétique saturée du genre *Prunus* ont permis de réaliser une analyse comparée des deux cartes. Des homologies ont été observées au sein des groupes de liaison respectifs. Aucune différence significative n'a été observée quant à l'ordre linéaire des marqueurs communs.

Mots clés : pêcher, carte de liaison génétique, *Prunus persica*, *Prunus ferganensis*, marqueurs moléculaires.

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Introduction

Molecular markers are an important tool for breeding selection, genotype identification, and studying the organization and evolution of plant genomes. Genome mapping is one of the most important applications of molecular markers. The advent of DNA-based markers has dramatically facilitated the mapping of several plant genomes in the last decade. The linkage between molecular markers and genes controlling important horticultural traits (either monogenic or polygenic) makes their introgression via marker-assisted selection (MAS) faster, and may be used as a first step towards saturation of the target region for positional cloning (Tanksley et al. 1995). Several classes of DNA-based markers are used for these purposes. Codominant highly reproducible and easily transferable markers, such as restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs), are the best possible choice, since they provide more genetic information than dominant mark-

ers and can easily be transferred to other mapping populations.

Peach (*Prunus persica* (L.) Batsch) is the second most important fruit crop economically in Europe, and Italy is the first in production among western countries. Peach breeding is time consuming, especially for fruit-specific characters, owing to an intergeneration period of not less than 3–4 years. The linkage between agronomic traits and molecular markers allows an early and efficient selection of traits long before they are expressed. Peach is a diploid ($2n = 16$) self-compatible autogamous species. The DNA content per nucleus is only 0.60 ± 0.03 pg (Baird et al. 1994)—about twice the size of that of *Arabidopsis thaliana*. Most western peach varieties originated from a few genotypes introduced into the U.S.A. in the nineteenth century from the South of China (“Chinese cling” type). For this reason peach cultivars are highly inbred and very low in genetic variability (Scorza et al. 1985). The relatively short intergeneration period, compared with other fruit crops, and the self-compatibility character make peach one of the best genetically characterized species of the genus *Prunus* (Hesse 1975; Monet 1989).

In the last decade, with the advent of DNA-based markers, genetic studies have been greatly facilitated and several genetic maps have been published for *Prunus*. Four of them, using peach (Rajapakse et al. 1995), peach × almond (Foolad et al. 1995; Joobeur et al. 1998), almond (Viruel et al. 1995), and sour cherry (Wang et al. 1998) progenies, were based mainly on RFLPs. The map of the interspecific F₂ cross ‘Texas’ × ‘Earlygold’ (T × E) was developed by the “European *Prunus* Mapping Project” (AIR-CT93-1585), a

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cooperative effort involving three partners (Joobeur et al. 1998). It is composed of 246 markers, with many loci in common with other *Prunus* maps and with two apple maps; owing to its high level of saturation, this map could be considered the reference map for the genus. Bošković et al. (1997) produced two maps composed only of isozymes using two cherry interspecific crosses. Other maps were constructed using random amplified polymorphic DNAs (RAPDs) as the predominant markers. These maps were developed using peach (Chaparro et al. 1994), peach × *Prunus davidiana* (Dirlewanger and Bodo 1994; Dirlewanger et al. 1996), and sweet cherry (Stockinger et al. 1996). Amplified fragment length polymorphisms (AFLPs) were mainly used to elaborate a map in peach rootstock progenies (Lu et al. 1998). Dirlewanger et al. (1998) constructed a linkage map in an intraspecific peach progeny with RFLPs, RAPDs, AFLPs and intermicrosatellite amplifications (IMAs).

The peach map presented here is composed of 109 loci that are mainly codominant (RFLPs and SSRs). To verify the maintenance of the linear order and to compare the genetic distance covered by the common loci, a subset of 32 loci in common with the T × E map was used in a comparative analysis between the two maps. Our objectives are to develop a saturated linkage map in peach, and to use it to identify molecular markers linked to traits of agronomic interest. Several quantitative trait loci (QTLs) have been identified, including *Sphaerotheca pannosa* resistance and fruit-related characters (Quarta et al. 2000). Molecular markers linked to these traits could be used in MAS in peach and other related species of *Prunus*.

Materials and methods

Plant materials

The peach selection IF7310828 ('J.H. Hale' × 'Bonanza') was crossed as female parent to an accession of *Prunus ferganensis* carrying a source of resistance to powdery mildew. A single F₁ plant, used as the source of pollen, was backcrossed to the peach selection IF7310828, to obtain a BC₁ population of 297 trees (P × F). Seventy random BC₁ individuals were used to construct the genetic map.

DNA extraction

Young unexpanded leaves were collected from the parents and from each BC₁ seedling and stored at -80°C until processed. Total genomic DNA was extracted according to the CTAB (cetyltrimethylammonium bromide) procedure described by Doyle and Doyle (1987), with few modifications. Three grams of leaf tissue was ground to a fine powder in liquid nitrogen. Half a gram of insoluble PVPP (polyvinylpyrrolidone) was added and the mixture extracted twice with chloroform-octanol (24:1). Before the second extraction, a volume of 10× CTAB (10% CTAB (w/v), 0.7 M NaCl), equal to 10% of the aqueous phase, was added. RNA was removed by digestion with RNase, and DNA was recovered by hooking. DNA concentrations were measured with a mini-fluorometer (TKO100, Hoefer Scientific, San Francisco, Calif.).

Marker analysis

All the markers were initially tested on the donor recurrent parent and the F₁ hybrid. Those detecting segregating polymorphisms were analyzed in the whole mapping population. Each marker was scored independently by three members of our group. Conflicting

data were re-examined and, in cases of disagreement, the most conservative option was taken.

RFLPs

Nine sources of DNA probes were used for RFLP analysis, as reported in Table 1. Ten of these were almond cDNAs previously sequenced and homologous to known genes (Joobeur et al. 1998). The FG genomic DNA library was obtained by digesting the total genomic DNA of *P. ferganensis* with *Pst*I. Fragments ranging from 1000 to 4000 bp were isolated from the gel and purified using the kit GeneClean II (Bio 101 Inc., La Jolla, Calif.) and then ligated into the plasmid pBluescript (Stratagene Cloning Systems, La Jolla, Calif.). Recombinant plasmids were used to transform *Escherichia coli* DH5- α strain following procedures in Sambrook et al. (1989).

DNA (4.5 μ g) was digested with five restriction endonucleases (*Bam*HI, *Dra*I, *Eco*RI, *Hind*III, and *Mva*I). Fragments were separated by electrophoresis in a 1% agarose gel (~1 V/cm). DNA Molecular Weight Marker II DIG-labeled (Roche Molecular Biochemicals) was included in each gel for molecular weight determination.

Denaturation, neutralization, and blotting were performed according to the Dig System user's guide for filter hybridization (Roche Molecular Biochemicals), using nylon neutral membranes (Hybond N, Amersham). DNA was fixed in the UV crosslinker Spectrolinker XL-1000 (120 mJ/cm²).

Probes were labeled by PCR amplification of the insert using 20 ng of each T3 and T7 primer. Probe amplification was done in a volume of 100 μ L containing 50 ng of template plasmid DNA; 100 μ M of each dATP, dCTP, and dGTP; 95 μ M of dTTP; 1.5 mM of MgCl₂; and 2 U of Taq DNA polymerase (Roche Molecular Biochemicals) in reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl). Digoxigenin-11-dUTP alkali-labile (5 μ M; Roche Molecular Biochemicals) was included in the PCR amplification mixture. Amplification was carried out in a thermal cycler (MJ Research PT100) with the following temperature profile: 2 min at 94°C; then 30 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C.

Membrane hybridization was performed according to the Dig System user's guide for filter hybridization in a hybridization oven at 68°C, using standard buffer (5× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% (w/v) SDS, 1% (w/v) blocking reagent, and 0.1% (w/v) *n*-lauroyl-sarcosine). The chemiluminescent substrate CPD-Star (Roche Molecular Biochemicals) was employed. Membranes were exposed to x-ray films (Kodak X-OMAT XAR-5) for 45 min at room temperature.

RAPDs

Reactions were prepared in a volume of 10 μ L containing 20 ng of template DNA, 0.2 μ M of 10-mer primer, 100 μ M of each dNTP, and 1 U of Taq DNA polymerase in reaction buffer (1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl). Amplification was carried out in a thermal cycler (Perkin Elmer 9600) with the following temperature profile: 95°C for 1 min; then 45 cycles of 95°C for 10 s, 37°C for 15 s, and 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis in 1.5% agarose gel (~2 V/cm) and stained with ethidium bromide. Operon primers (Operon Technologies, Alameda, Calif.) from kits A, C, G, H, I, J, K, L, P, V, and AD were tested.

SSRs

Microsatellites, obtained from the University of Udine (Italy), were scored in the BC₁ progeny. Their segregation was reported in two previous papers (Cipriani et al. 1999; Testolin et al. 2000). PCR amplification was done according to Testolin et al. (2000) in a Perkin Elmer 9600 thermal cycler, scaling down to a total volume

of 10 μ L. PCR products were separated in 3% MetaPhor (FMC Bioproducts) agarose gel in 0.5 \times TBE (1 \times TBE: 90 mM Tris-borate, 2 mM EDTA, pH 8.0) (~3 V/cm) and then stained with ethidium bromide.

Traits

Several segregating characters were scored in the progeny (Quarta et al. 1998). Two of them, flesh adhesion (*F/f*) and leaf glands (*E/e*), were monogenic and were included in the linkage analysis.

Linkage analysis

As a result of using non-inbred parents, five types of single-locus segregation arose in the BC₁ population: 1:1 σ^2 , 1:1 φ , 1:2:1, 3:1, and 1:1:1:1 (Table 2). Chi-square goodness-of-fit tests were done with the JMSLA module of JoinMap 2.0 (Stam and Van Ooijen 1995). Two software packages, JoinMap 2.0 and MAPMAKER/EXP v. 3.0 (Lander et al. 1987), were employed to handle our data. The Kosambi function (Kosambi 1944) was used to convert recombination units into genetic distances.

Since most markers (70) segregated in a 1:1 σ ratio, the backcross model of MAPMAKER was used at first for mapping only these markers. Linkage groups were established with the "group"

command at a logarithmic odds (LOD) score of ≥ 3 and a recombination fraction of 0.3. Once groups were established, only tightly linked loci (LOD ≥ 5 and recombination fraction = 0.2) were analyzed to set up a robust framework for each linkage group. These loci were ordered using the "order" command, with the "multipoint criteria" set to the strict threshold of LOD ≥ 5 for the first round of ordering and LOD ≥ 3 for the second round. The remaining markers were added to these frameworks using the "try" and "ripple" commands. Small groups (up to four loci) were ordered with the "compare" command. After mapping, the "error detection" command of MAPMAKER was employed and possible errors were re-

guarantee the best-order solution (Stam and Van Ooijen 1995; Qi et al. 1996). The JMMAP module was used with a LOD and recombination threshold of 0.5 and 0.45, respectively. Markers that increased by three the goodness of fit (χ^2) of the map ("jump" threshold ≥ 3) were removed and added later one by one. They were tentatively placed only if they did not give rise to a rearrangement of the map obtained without them. Otherwise, they were not assigned to a specific position in their group but only listed as belonging to it.

Results

We tested 388 probes (277 genomic DNA and 111 cDNA, including the known genes) to detect polymorphisms among the parents. One hundred and ten probes showed polymorphisms (28.4%). The percentage of polymorphism detected by cDNA (26.1%) and genomic DNA probes (29.2%) was almost the same. Sixty-nine probes showed polymorphisms with more than one restriction endonuclease. Of 110 polymorphic probes, some did not segregate in the BC₁ and others, mainly cDNAs with low molecular weight, yielded a very low signal and were discarded. Seventy-six probes, 57 genomic DNA and 19 cDNA, were analyzed for linkage in the BC₁ and produced 81 RFLPs (Table 2). Three clones (AC33, FG51 and FG189) detected two loci each and one (AG8) detected three loci. Two polymorphic cDNA probes corresponded to the known genes phosphoglycerate mutase (*PglI*) and jasmonic acid protein (*PijI*) previously mapped by Viruel et al. (1995) and Joobeur et al. (1998).

Ninety-nine Operon primers were tested in the parents. Eighty were randomly chosen, while 19 had been previously mapped in almond (Joobeur et al. 2000). Thirty-one Operon primers showed polymorphism among the parents (31.3%), but most of those expected to segregate 3:1 were discarded, being poorly informative. From the 13 scorable primers showing useful segregation in the progeny, we obtained 18 RAPD markers (Table 2).

In two previous papers (Cipriani et al. 1999; Testolin et al. 2000), 26 SSRs were analyzed in 16 individuals of the BC₁ progeny, and segregation was demonstrated for 17 of them (65.4%). They were scored in all the individuals and mapped (Table 2).

The segregation ratio of the markers is reported in Table 2. Seventy loci segregated as in a true backcross (1:1 σ), while 30 were F₂ type (1:2:1 and 3:1). The 1:1 η segregation type, informative for the recurrent parent, was less represented (17 loci). Only one locus with three alleles, segregating in a 1:1:1:1 ratio, was found. The monogenic traits flesh adhesion (*F/f*) and leaf glands (*E/e*) segregated 3:1 and 1:2:1, respectively (Quarta et al. 1998).

In total, we scored 118 markers in the BC₁ progeny. One hundred and thirteen markers coalesced into 10 linkage groups (Fig. 1). Five markers (three RFLPs and two RAPDs) remained unlinked (4.2%). Ten markers increased the goodness of fit (χ^2) of the map ("jump" value) to a value higher than three. Four of them (AC33b, FG54a, FG201, and PC34) gave rise to important rearrangements in the group order and, thus, were not placed on the map but listed at the end of their linkage group. The remaining six markers (AG105a, CC122a, FG240, OPAD16a, OPH3a, and UDP98-415) were tentatively placed. The map is composed of 109 loci: 74 RFLPs, 17 SSRs, 16 RAPDs, and two morphological traits.

The linkage groups were named according to the nomenclature adopted by Viruel et al. (1995) and Joobeur et al. (1998), because of a subset of loci held in common. These anchor loci allowed us to assign two small linkage groups, each composed of three loci, to G1 and G8; we named them G1A and G8B, according to their orientation in the T \times E map. The map covers 521 cM of the peach genome. The average distance between adjacent markers is 4.8 cM but, if only one marker is taken into account in regions with cosegregating markers, the average distance becomes 5.1 cM. Markers are unevenly distributed among linkage groups, with the average distance between markers ranging from 2.8 cM in G2 to 7.7 cM in G3 and G8B. The density of markers is very high in the 26- to 58-cM region of G2 (1.8 cM/marker). Two large gaps, longer than 20 cM, are located in G1B and G7. Thirteen more gaps ≥ 10 cM are found: four in G3, two in G1B and G8A, and one each in G2, G4, G6, G7, and G8B.

Flesh adhesion (*F/aMa(10)-315(cM)-315(areM)-315(7P-38ov)[(aM.2(b*

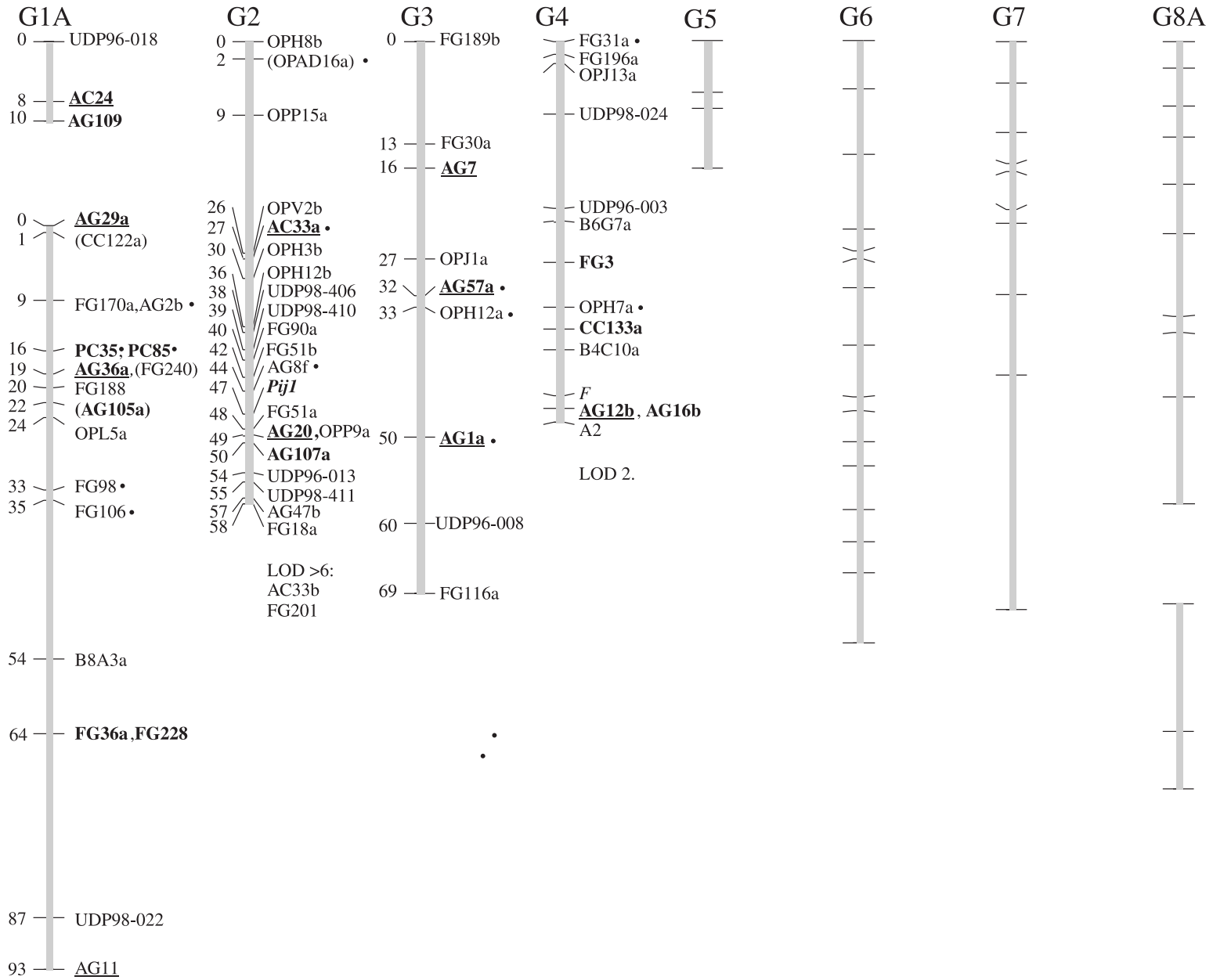


Table 3. Comparisons among homologous distances in the P × F and T × E maps elaborated with JoinMap (JM) and MAPMAKER (MM).

	Analyzed length				No. of common loci	No. of groups analyzed	Paired <i>t</i> test	
	P × F map		T × E map				<i>t</i>	<i>P</i>
	Distance (cM)	%	Distance (cM)	%				
P × F (JM) vs. T × E (MM)	227	43.6	199	40.5	32	8	0.87	0.205
P × F (JM) vs. T × E (JM)	227	43.6	167	39.0	32	8	1.91	0.049
P × F (MM) vs. T × E (MM)	128	24.0	77	15.7	19	4	1.36	0.13

comparison of the two MAPMAKER maps, in which a lower proportion of the map distances was involved, revealed no significant differences, while in the case of distances calculated with JoinMap 2.0, the P × F map was significantly longer.

A few clones (AG2, AG8, AG60, FG209, and MC011) detected loci other than those found in the T × E map. AG8 identified three loci in both the T × E and in P × F maps, but in different groups.

Discussion

The rate of RFLP polymorphism (28.4%) was higher than the values of about 19 and of 22.8% obtained in other peach × peach progenies by Rajapakse et al. (1995) and Dirlewanger et al. (1998), respectively. The greater genetic distance between the donor and recurrent parents and the way in which we chose the probes to be tested may explain the increased rate of polymorphism. In fact, we tested the FG probes randomly, while all the other probes were chosen, since they had previously been mapped in other *Prunus* progenies. If we only consider the FG clones, the rate of RFLP polymorphism was 22.3%, a value not much different from those obtained by Rajapakse et al. (1995) and Dirlewanger et al. (1998). The rate of RFLP polymorphism in almond × peach progenies (Foolad et al. 1995) was higher, as expected in an interspecific cross between two distant species.

Similarly, RAPD markers showed differences in polymorphism depending on how they had been chosen. In the group of randomly chosen primers, the proportion of polymorphism was 23%, close to the value (17%) observed in other peach progeny (Dirlewanger et al. 1998), but in the group of primers already mapped in almond it was 68.4%.

SSRs were highly polymorphic (65.4%), confirming their power to detect variation among individuals. Moreover, their codominant nature and single-locus behavior make them very attractive for genome analysis.

Most of the loci segregated in a 1:1 ratio and were informative for the F₁ hybrid (Table 2). In fact, peach is a self-pollinating species with a high level of inbreeding (Scorza et al. 1985) and many loci are expected to be homozygous. Actually, 23 of the RFLPs (28.4%) and nine of the SSRs (52.9%) were homozygous in both the donor and the recurrent parent. Moreover, when a probe detected polymorphisms with more than one restriction enzyme, we chose the combination that was expected to segregate in a 1:1 ratio, informative for the F₁ parent. Finally, markers heterozygous for the same alleles in both the parents and the hybrid

were always discarded, as all of them had the same banding pattern and could not be recognized as polymorphic.

Our map shows 10 linkage groups, two more than the chromosome number of *Prunus* ($x = 8$). Viruel et al. (1995) and Joobeur et al. (1998) found eight groups. The reason for the absence of linkage among groups belonging to the same chromosome was probably not the poor marker coverage of the region but the low level or absence of informativity between loci. This lack of informativity and the difficulty of guaranteeing the best order with JoinMap (Stam and Van Ooijen 1995; Qi et al. 1996) forced us to construct a framework with MAPMAKER using those loci informative for the F₁ hybrid. This framework was then used as fixed in JoinMap 2.0. Running JoinMap without the “fixed order” option, we observed differences in marker order in G1B, G7, and the very dense region of G2; the map obtained with the “fixed order” procedure was accepted, owing to a better goodness-of-fit value (χ^2) for these groups.

The proportion of skewed segregations (18.5%) was much lower than that found by Joobeur et al. (1998) and Foolad et al. (1995) using almond × peach progenies (46 and 37%, respectively). Using a peach × peach progeny, Dirlewanger et al. (1998) found a very low rate of skewed segregation (2%). Our value was similar to that (18%) reported by Dirlewanger et al. (1996), when they used a progeny from two closely related species (*P. persica* and *P. davidiana*). In our progeny, no plants were lost after planting, thus the distortions could not be attributed to selection in the field, as was found by Foolad et al. (1995) and Joobeur et al. (1998). The selection probably occurred at the pre- or post-zygotic level before seed germination or in the early stages of plant development against lethal or sublethal genes closely linked to the markers under selection. Similarities were observed in the regions of the P × F and T × E maps with skewed segregations. In G1, the *P. persica* alleles were favored in both maps, while in G3 and G4, they were selected against. In the region at the end of G6, the direction of the distortion was divergent, being toward the *P. persica* alleles in the T × E map and against them in the P × F map. In the T × E map, this distortion was attributed to the almond self-incompatibility gene (*Si*) mapped in this region (Ballester et al. 1998; Joobeur et al. 1998). However, peach is a self-compatible species and the selection against the recurrent parent alleles in G6 cannot be ascribed to the self-incompatibility reaction. In the T × E map, almost all the loci in G5 and G7 did not have skewed segregations; similarly, in the P × F map, all the loci in these groups followed the Mendelian ratio.

Locus order was well preserved between the P × F and T × E maps, except for two loci that were in inverted order in

G1. The small number of seedlings analyzed in the two maps could explain this incongruity, as the order cannot be defined with precision for tightly linked loci.

The genetic distances covered by common loci were always longer in our map than in the T × E map, however the maps were calculated. The paired *t* test values were not significant in the comparison of the maps produced by different programs or in the comparison involving the two maps elaborated with MAPMAKER. In contrast, the paired *t* test was significant when distances for the two maps were calculated with JoinMap. It is reported in the literature (Qi et al. 1996; Van Ooijen et al. 1994) that JoinMap produces shorter maps than MAPMAKER. When we compared the P × F maps elaborated separately with the two programs (MAPMAKER map not shown), the paired *t* test pointed out that the distances obtained with JoinMap were significantly shorter (66 loci in common; *t* = 3.51, *P* = 0.005). The lack of significance in the test involving the two MAPMAKER maps could be explained by the shorter distances compared and the smaller number of common loci distributed in only four groups. Thus, it is likely that the P × F map is significantly longer than the T × E map, suggesting a different rate of recombination in the two crosses. The genetic diversity of the individuals involved in the crosses (peach and almond in the T × E map and two closely related species in the P × F map) may explain this phenomenon. The reduction of recombination in interspecific hybrids has been observed in other species (Gebhardt et al. 1991; Causse et al. 1994).

The density of markers was similar to or higher than that in other peach maps (Rajapakse et al. 1995; Dirlewanger et al. 1998; Lu et al. 1998) and half of that found in the T × E map (5.1 vs. 2.7 cM/marker). Fifteen gaps ≥10 cM were present (2.9 gaps of ≥10 cM per Morgan); this is 3.6 times as many as were found in the T × E map, which can be considered to be saturated. The total length was similar in the two maps (521 vs. 491 cM). The only exception was G5, in which a consistent part of the group was clearly missing in the P × F map. These data suggest that our map probably covers most of the peach genome, with a good level of saturation corresponding to about half the T × E map.

The current map is composed mainly of codominant markers (RFLPs and SSRs) that are easily transferable to other populations. The number of loci in common with other *Prunus* maps enables one to perform synteny studies and eventually, to construct a consensus map for the genus. Our map is currently used to identify agronomic traits of interest, such as disease-resistance and fruit-related characters. Markers tightly linked to these traits will be used to facilitate their introgression and maintenance in breeding lines through MAS.

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