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Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers

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Abstract Linkage maps for the apple cultivars 'Prima' and 'Fiesta' were constructed using RFLP, RAPD, isozyme, AFLP, SCAR and microsatellite markers in a 'Prima' × 'Fiesta' progeny of 152 individuals. Seventeen linkage groups, putatively corresponding to the seventeen haploid apple chromosomes, were obtained for each parent. These maps were aligned using 67 multi-allelic markers that were heterozygous in both parents. A large number of duplicate RFLP loci was observed and, in several instances, linked RFLP markers in one linkage group showed corresponding linkage in another linkage group. Distorted segregation was observed mainly in two regions of the genome, especially in the male parent alleles. Map positions were provided for resistance genes to scab and rosy leaf curling aphid (*Vf* and Sd_1, respectively) for the fruit acidity gene *Ma* and for the self-incompatibility locus *S*. The high marker density and large number of mapped codominant RFLPs and some microsatellite markers make this map an ideal reference map for use in other progenies also and a valuable tool for the mapping of quantitative trait loci.

Key words *Malus pumila* Mill · Molecular linkage map · Marker-assisted selection · Fruit tree breeding · Outbred progeny

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Introduction

Although apple (*Malus pumila* Mill.) has been cultivated for centuries and is one of the main fruit tree species in the world, genetic studies and breeding have always been hampered by the long generation cycle, the space, time and cost involved in screening and maintaining populations, the high chromosome number ($2n = 34$) and its outbreeding mode of reproduction. These same considerations have stimulated interest in

molecular genetics, since molecular markers provide tools to detect genes for economically important traits and for early selection of these traits in breeding programmes.

Backcrossing is an unrealistic option for an outcrossing tree species due to the long juvenile period and self-incompatibility. Therefore, genetic analysis in apple is typically performed in the full-sib progeny of a single cross, which is also the base population for selection in breeding. Both parents of a cross are expected to display a high level of heterozygosity, thereby allowing markers to be found that are heterozygous in one or both parents. This type of analysis has been named double pseudo-testcross or two-way pseudo-testcross (Hemmat et al. 1994; Grattapaglia and Sederoff 1994). This terminology may, however, be confusing, since markers with a 3 : 1, 1 : 2 : 1 and 1 : 1 : 1 : 1 type of segregation (if both parents are heterozygous) can be used in addition to markers with a 1 : 1 segregation (if one parent is heterozygous).

Various types of markers are being used in studying apple genetics. Markers for important genes have already been provided by isozymes: *AAT-1* (*GOT-1*) was shown to be linked to the self-incompatibility locus *S*, *ACP-1* and *ENP-1* to the pale-green lethal gene *l*, *LAP-2* to the mildew resistance gene Pl_w and *PGM-1* to the scab resistance gene *Vf* (Manganaris and Alston 1987; Manganaris and Alston 1988; Manganaris and Alston 1992; Manganaris et al. 1994). More recently, random amplified polymorphic DNA (RAPD) markers have been used to find closer linkage to *Vf* (Yang and Krüger 1994; Koller et al. 1994; Durham and Korban 1994; Gardiner et al. 1996; Tartarini 1996; Yang et al. 1997a,b). RAPD markers have also been found for the mildew resistance gene Pl_1 (Markussen et al. 1995), R_f for fruit skin colour (Cheng et al. 1996), *Tb* for terminal bearing, *Rbb* for initial bud break, *Rs* for rootsucker formation (Weeden et al. 1994) and *Co* for columnar tree habit (Hemmat et al. 1997). Linkage of restriction fragment length polymorphism (RFLP) markers to *Vf* and Sd_1 for resistance to rosy leaf curling aphid was reported by King et al. (1998) and Roche et al. (1997a), respectively. Some of these markers have been transformed successfully into sequence characterized amplified regions (SCARs) or cleavage amplified polymorphic sites (CAPSs), which are more efficient than RAPDs or RFLPs for selection in apple breeding (Markussen et al. 1995; Gianfranceschi et al. 1996; Yang and Korban 1996; Yang et al. 1997a,b; Roche et al. 1997b; S. Tartarini et al. personal communication). In a recent paper, the characterization of microsatellites in apple has also been reported (Guilford et al. 1997). One microsatellite marker was developed from a marker containing a simple sequence repeat (SSR), linked to the *Co* gene for columnar tree habit (Hemmat et al. 1997).

A first molecular genetic linkage map for apple covering over 400 markers was published by Hemmat et al. (1994). Fifteen homologous pairs of linkage groups were identified using markers that were heterozygous in both parents. Additionally, nine and six single linkage groups were formed for the respective parents. Since this map is mainly based on RAPD markers, it cannot easily be transported to other progenies. The relatively small progeny size is the likely cause that several parts of linkage groups remained unlinked. Additional RAPD-based maps were constructed for three cultivars using two larger progenies (Conner et al. 1997), and homologies between all three maps were established for thirteen linkage groups. Isozyme and morphological markers also allowed the identification of six linkage groups homologous to the map of Hemmat et al. (1994). Since these maps are mainly based on RAPD markers, their usefulness in new progenies is very limited. Conner et al. (1997) therefore recommended using additional codominant markers.

This paper presents the construction of a linkage map containing more than 200 RAPD, isozyme, RFLP, SSR and amplification fragment length polymorphism (AFLP) markers. The map is one of the results of the European Apple Genome Mapping Project which was started in 1993 to study the genetics and genotype-by-environment interaction of a wide range of resistance, tree habit, fruit quality and fruit production characters (King et al. 1991; King 1996).

Materials and methods

Plant material

A cross between the apple cultivars 'Prima' and 'Fiesta', using 'Fiesta' as the pollen parent, was made at CPRO-DLO, the Netherlands, in 1988. The petals of unopened 'Prima' flower buds were removed and, after pollination, the clusters were bagged to prevent foreign pollination. 'Prima' is a scab resistant cultivar carrying the *Vf* gene from *Malus floribunda* 821, and selections derived from 'Prima' are used in breeding programmes throughout the world to transfer this resistance. 'Fiesta' (syn. 'Red Pippin'), derived from a cross between 'Cox's Orange Pippin' and 'Idared', has a high productivity and good fruit quality and shelf life. The germination rate of the seeds was 73%. A seedling population of 161 trees was established and used as a source for propagation and leaf material for marker screening. Replicate sets of trees were propagated by grafting onto M27 rootstock. One set of grafted trees and the original seedlings were established in the field at CPRO-DLO. Other (sub-)sets were distributed to six sites in Europe (HRI East Malling, UK; HRI Wellesbourne, UK; IZZ Ahrensburg, Germany; INRA Angers, France; DCA Bologna, Italy and NAGREF, Naoussa, Greece) for DNA and protein extraction and phenotypic observations. On site, trees were re-propagated if this was necessary for resistance tests. The population was screened in the greenhouse and field for resistance to diseases and pests, fruit quality and fruit production parameters and tree habit.

During marker analyses, mostly from isozyme and RFLP markers, it became evident that eight individuals in the mapping population were outcrosses. In addition, isozyme and RFLP analyses suggested that one individual was a triploid. These nine individuals were excluded from linkage analysis.

Markers

Notation

The notation of segregating markers follows Maliepaard et al. (1997) and uses different characters to denote different alleles of a marker locus including '*0*' for a null-allele. Left of the '×' is the genotype of the mother, at the right the genotype of the father. Segregation types $ab \times cd$ and $ab \times ac$ are used for markers segregating into four marker phenotypes in the progeny; $ab \times ab$ is used for markers when parents are both heterozygous for the same two alleles and the expected segregation is 1 : 2 : 1 as in an F_2 of a selfing species. If a dominant marker is present in both parents and segregates in the progeny with an expected 3 : 1, the notation $a0 \times a0$ is used for segregation of the fragment. Segregation types $ab \times aa$ and $aa \times ab$ are used for markers which are heterozygous in the female and male parent, respectively.

Linkage groups are indicated with 'L' for linkage group followed by a number. 'Prima' and 'Fiesta' homologues of a pair of linkage groups are indicated with 'Pr' and 'Fi'. For example, L01-Pr refers to the Prima homologue of linkage group L01.

RAPDs

Over 600 decamer primers were screened for markers that were polymorphic between 'Prima' and 'Fiesta'. Primers were obtained from Operon (OP), Genosys (GE) and the University of British Columbia (UBC). Initial experiments were carried out to determine the reproducibility of RAPD marker generation and scoring between four laboratories. Identical reaction conditions and DNA samples were used, and all reactions were carried out in Hybaid OmniGene thermal cyclers (tube control). Following the comparison of replicated experiments, it was found that primers which produced many or faint bands were inherently less reproducible. Primers producing a few very bright bands were used for systematic and reproducible mapping. It was also found preferable to select primers that produced one or more non-polymorphic bands flanking the polymorphic bands in the track, as these acted as internal controls of the efficiency of the amplification reaction (King 1994).

RFLPs

A cDNA library was made by Invitrogen Ltd from leaf material collected from M27 rootstock trees. Clones from this library are indicated with MC (*Malus* cDNA). In addition, apple cDNA clones were obtained from a library constructed by M. Lay Yee and selected as RFLP probes for apple at HortResearch. These are indicated as 'LY'. Genomic clones from two libraries constructed at Ahrensburg were used and are indicated with 2B10, 2B11 etc. (one as 7BC7) and 'MRC', respectively. At HRI, twelve genomic clones from a sub-genomic library developed by S. Tartarini were used, indicated with 'MH' (*Malus Hin*dIII). Known *Prunus* gene sequences Oleosin (EMBL accn: X78118) and Extensin (EMBL accn: X65718) as well as cDNA and genomic clones from almond ('AC' and 'AG', respectively) were also screened (Viruel et al. 1995). Probes designated LBA from a *Prunus avium* cDNA library were kindly supplied by Dr. P. Hand, HRI. Known gene sequences were also used as clones in RFLP analysis: pB610 (homologous to TPI = triose phosphate isomerase; Dr. G.S. Ross, personal communication), pAP4 (EMBL accn: X61390, ACC synthase), pAP79, pAP260 (kindly obtained from Dr. N. Weeden, Cornell University), pADH32 (kindly obtained from Dr. G.S. Ross, HortResearch, New Zealand) and pS6 (EMBL accn: D11080; NADP-dependent D-sorbitol-6-phosphate dehydrogenase; kindly obtained from Dr. S. Yamaki, Nagoya University, Japan). One clone also used for RFLP analysis was M18, kindly obtained from Dr. L. Gianfranceschi. This is a cloned fragment from RAPD marker OPM-18–0900, closely linked to *Vf*, from which Gianfranceschi et al. (1996) developed a CAPS marker

At CPRO-DLO, DNA extraction, Southern blotting, clone labeling and hybridization were as in Roche et al. (1997a). Clones were first tested on the parents and a subset of ten progeny plants using six restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII and *Xba*I). Clones were selected for the analysis of the entire progeny if the banding pattern fitted an $ab \times ab$-, $ab \times ac$-, or $ab \times cd$-type of segregation or if multiple markers could be scored. In many cases segregation of two marker loci was evident, but both loci could not always be scored using a single restriction enzyme. Therefore, in a number of cases, two restriction enzyme/probe combinations were scored. In a modification to the procedure followed at CPRO-DLO, the enzymes used at HRI were *Eco*RI, *Xba*I and *Dra*I, whilst IZZ used *Eco*RI and *Hin*dIII.

In total, 245 clones were used to screen the parents and a small subset (usually ten plants) of progeny for segregating bands with a number of restriction enzymes. Ninety-two clones were used to screen the larger progeny.

Isozyme markers

Isozyme analysis was performed on extracts from fresh leaves, in acrylamide and starch gels, according to protocols described in Chevreau and Laurens (1987) and Manganaris and Alston (1987). Staining was performed according to Wendel and Weeden (1989) except for catechol oxidase (CO) which was stained according to Schwennesen et al. (1982). Fifty-five markers from 21 enzyme systems (AAT 2.6.1.1, ACO 4.2.1.3, ACP 3.1.3.2, ADH 1.1.1.1, CO 1.10.3.-, DIA 1.6.99.-, ENP 3.4.9.9, EST 3.1.1.-, FDH 1.2.1.2, GDH 1.4.1.2, IDH 1.1.1.42, LAP 3.4.11.1, MDH 1.1.1.37, ME 1.1.1.40, PGD 1.1.1.44, PGI 5.3.1.9, PGM 5.4.2.2, PRX 1.11.1.7, SKD 1.1.1.25, SOD 1.15.1.1, TPI 5.3.1.1-enzyme codes are followed by EC numbers-) were screened in the progeny.

Microsatellite markers

Four microsatellite markers developed by HRI were used to screen the progeny. A further five microsatellite markers were obtained from HortResearch (Guilford et al. 1997). One microsatellite marker, based on the SSR present in a marker for the *Co* gene, was also used (Hemmat et al. 1997). Using the specific primers and optimized annealing temperatures, we carried out polymerase chain reaction (PCR) using [γ-^{33}P] ATP-labeled forward primers. The products were separated on 7.5 *M* urea denaturing 6% polyacrylamide gels, which were then dried and visualized following exposure to Fuji X-ray film.

AFLP markers

AFLP (Amplified Fragment Length Polymorphism) is a molecular-marker technique based on selective PCR amplification of restriction fragments (Vos et al. 1995). An aliquot of 500 ng genomic DNA was digested with restriction enzymes *Eco*RI and *Mse*I. Restriction fragments were ligated with double-strand adapters, and a pre-amplification was done using the appropriate primers with one added selective nucleotide. The reaction mix was diluted 1/40, and 10 µl was used for the final amplification with two primers each having three selective nucleotides. One of these primers was end-labeled with γ-^{33}P. As an initial study of the use of AFLP markers in apple, one primer combination was used: 5′ GAC TGC GTA CCA ATT CAC A 3′ (E35) and 5′ GAT GAG TCC TGA GTA ACA A 3′ (M47); the three selective nucleotides are underlined. Amplification products were separated on 5% denaturing polyacrylamide gels.

The gels were dried and exposed to Kodak XOMAT AR X-ray film for 10–14 days at room temperature. Segregating AFLPs were coded as E35/M47/P1 to E35/M47/P6 for 'Prima' fragments and E35/M47/F1 and E35/M47/F2 for 'Fiesta' fragments in order of decreasing fragment size. One segregating fragment present in both parents was labeled E35/M47/PF1. Eighty-two plants were scored for presence or absence of these fragments.

SCAR marker

The BC226 SCAR, which was found to be linked to a gene for fruit skin colour, was used with no modification (Cheng et al. 1996).

Linkage analysis

Single-locus analysis, grouping of markers and mapping were performed with JoinMap version 2.0 which permits linkage analysis in outbred progenies involving markers with different segregation types (Stam 1993; Stam and Van Ooijen 1995). The Kosambi mapping function was used for the calculation of map distances. Markers were first divided into linkage groups using a LOD score threshold of 4.0. This LOD score calculated in JoinMap version 2.0 is based on the chi-square test for independence of segregation and is different from the usual LOD score in linkage analysis. The latter LOD score is affected by distorted segregation, while the test of independence is not. In this paper the LOD score is the JOINMAP 2.0 LOD score unless specified differently. After being divided into linkage groups, markers were separated for the individual parents, using where possible only the alleles from that particular parent to calculate recombination frequencies. Obviously, this was not possible for $ab \times ab$- and $a0 \times a0$-type markers. The $a0 \times a0$-type markers were assigned to linkage groups but not used in the construction of the linkage map since such markers contribute little information to the map and recombination frequency estimates obtained with such markers are typically inaccurate (Maliepaard et al. 1997).

In a few cases a marker was discarded during the mapping stage if its presence caused inconsistencies in the map. Such markers were identified by discrepancies (for multiple marker intervals) between the original distances with the final map distances after integrating all markers of a linkage group.

Monogenic traits

Scab resistance was evaluated in field and glasshouse tests in 1993, 1994 and 1995 at sites in France, Germany, Greece, Italy, the Netherlands and the UK. A clear bimodality and a strong agreement with respect to the resistance classification was observed in a number of data sets. These observations fitted a 1 : 1 segregation, as was expected for *Vf*, which is a single, dominant gene. Cluster analysis was then used to combine data sets and divide the progeny into two clusters representing consensus resistant and consensus susceptible plants (King et al. in press). Using this consensus score, we mapped the *Vf* gene for resistance to apple scab (*Venturia inaequalis* Cke.) as a single, dominant gene.

Fruit acidity was evaluated using bromocresol and pH indicator paper to distinguish between fruits with higher and lower acidity. pH values lower than 3.8 were considered to indicate the presence of the *Ma* gene; genotypes with fruit pH values greater than 3.8 were considered to be of the *mama* recessive genotype (Visser and Verhaegh 1978). The parents were assumed to be heterozygous *Mama*. In total, 140 genotypes were tested at one or more of three sites.

The gene Sd_1 for resistance to two biotypes of rosy leaf curling aphid (*Dysaphis devecta* Wlk.), present in 'Fiesta', was mapped on the basis of field and glasshouse resistance tests at HRI East Malling. The tests and mapping of this gene have been described in Roche et al. (1997a).

For mapping the self-incompatibility locus, we screened the parents using the allele-specific primer method described by Janssens et al. (1995); this method indicated the 'Fiesta' alleles to be S_3 and S_5 and 'Prima' alleles to be S_2 and an undetermined *S* type. Further investigation by W. Broothaerts (personal communication) revealed the undetermined allele to be S_{10} and allowed the progeny to be scored with an additional allele-specific marker, thereby detecting all four alleles. In addition to this method, an RNase assay was performed. RNase activity has been associated with the self-incompatibility reaction and was detected following electrophoretic separation of stylar extracts (Bošković and Tobutt 1996). For the assay, flowers were collected from the field at the late-balloon stage. Stigmas were dissected and styles collected in microfuge tubes. Stylar extracts were prepared and the protein products were separated by gel electrophoresis. RNase alleles were detected following the methods described by Bošković and Tobutt (1996). Four alleles segregated in the population. Of 51 individuals tested with both the RNase and DNA allele-specific assays, there was cosegregation for 50 individuals. For one individual, the DNA assay indicated an S_2S_5 genotype, whilst the RNase assay indicated $S_{10}S_5$. For the same set of 51 individuals there was complete cosegregation between the RNase assay and *AAT-1*.

Results

Marker segregation

RAPD markers

In total, 168 RAPD markers from 88 primers were scored. Thirteen markers were present in both parents, and an $a0 \times a0$ (3 : 1)-type of segregation was assumed. However, five of these markers were discarded due to difficult reproducibility and strongly skewed segregation. Two of the markers were scored on a limited set of progeny, and no LOD scores larger than 4.0 were found. Therefore, only six of the $a0 \times a0$ segregating markers could be assigned to linkage groups (Fig. 1). Eighteen RAPD markers with an $ab \times aa$- or $aa \times ab$-type of segregation were removed from the analysis for different reasons (strong segregation distortion, nonrandom distribution of recombinants with other markers, interaction with other markers from the same primer). Four more RAPD markers were discarded during mapping because of inconsistencies in recombination frequency estimates with other markers. In the end, 133 RAPD markers were positioned on the linkage map.

One primer, OPT-09, generated two bands with a codominant pattern of segregation: there were no individuals for which both bands were absent. The segregation of the combined marker did not deviate from the expected 1 : 2 : 1 ratio. Although this marker could be assigned to linkage group L05, including this marker in the map resulted in inconsistencies with other markers and the marker was therefore discarded. Another primer, OPAC-15, generated two bands in the female parent (sizes 2000 and 2050 bp), of which either one or the other was always present in the progeny, thus behaving as alleles.

Markers OPAE-01 $\widehat{1210}$ and OPAE-01 $\widehat{1190}$ were mapped to the 'Prima' and 'Fiesta' homologues,

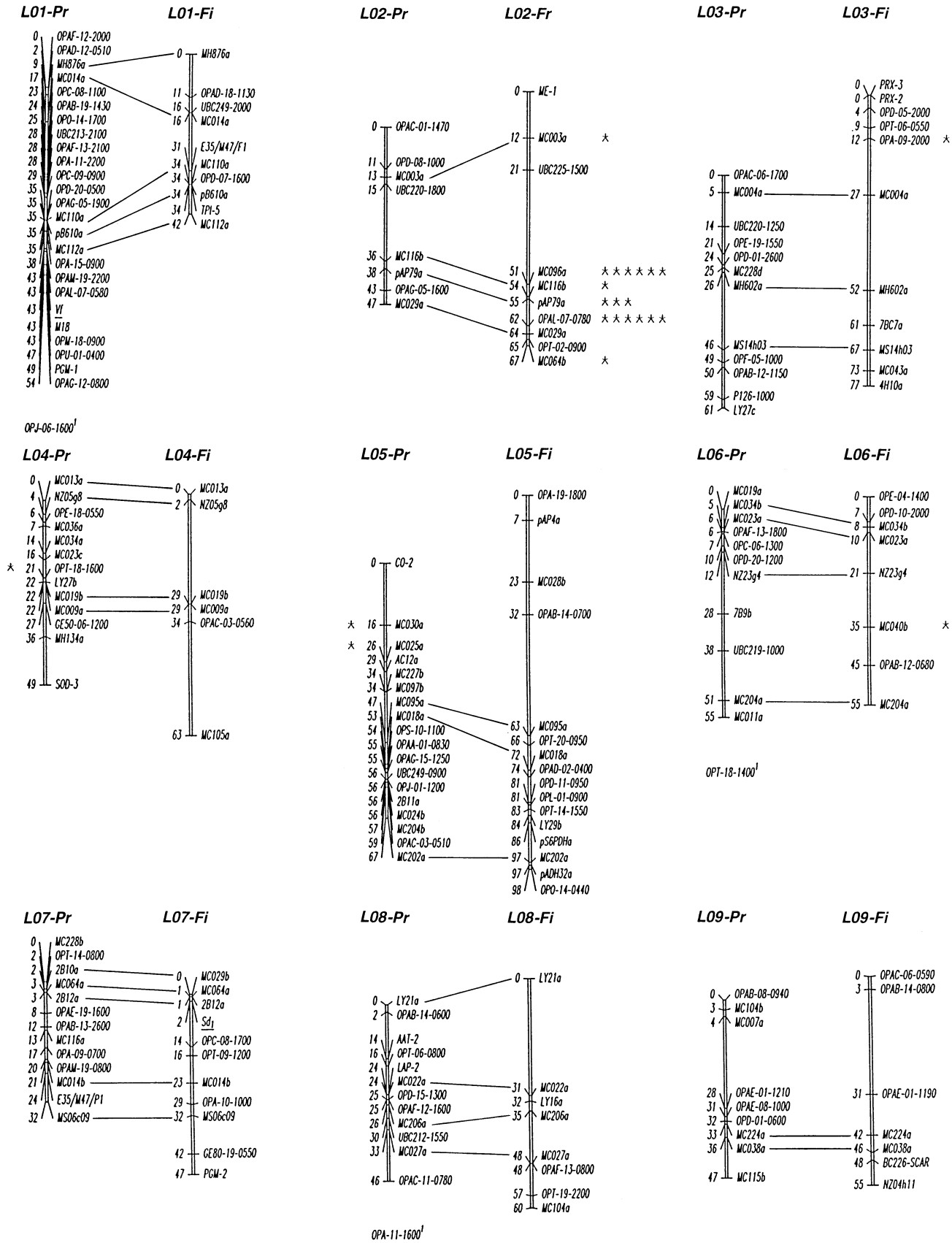

Fig. 1 See page 65 for legend

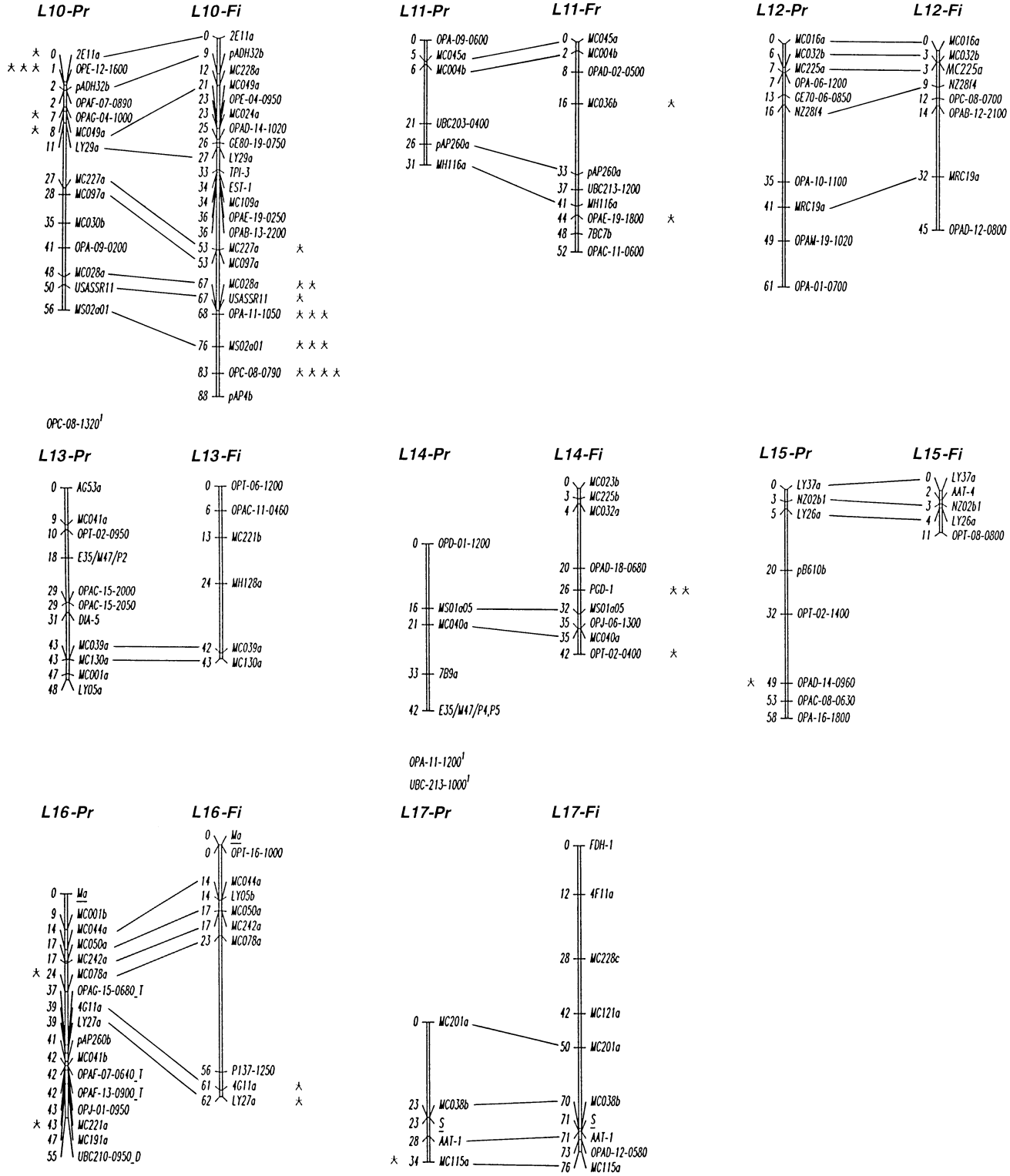

Fig. 1 Genetic linkage map of apple. 'Prima' (*Pr*) and 'Fiesta' (*Fi*) linkage groups are numbered from *L01* to *L17*. Allelic bridges are indicated by *lines* connecting *Pr* and *Fi* linkage groups. *Asterisks* indicate distorted segregations of markers (chi-square test). $*P = 0.05$, $**P = 0.01$, $***P = 0.005$, $****P = 0.001$, $*****P = 0.0005$, $******P = 0.0001$. [1]*a0* × *a0* segregating markers were assigned to linkage groups, but no map positions were determined

respectively, of linkage group L09 and possibly also represent two alleles of a single locus.

RFLP markers

In total, 124 RFLP markers from 86 probes were mapped. Thirty-seven probes generated one or more markers including one with an $ab \times cd$ or an $ab \times ac$ segregation type, seventeen generated one or more markers including one with an $ab \times ab$ type of segregation. Thirty-two probes generated one or more markers with an $aa \times ab$- or an $ab \times aa$- type of segregation. Two probes (LY27 and MC023) yielded markers for three, another (MC228) for four loci.

Microsatellite markers

Ten SSR markers were used in this study. Each generated only a single marker locus; all ten markers were mapped. With the exception of two markers which are linked on L10, all SSR markers were mapped on different linkage groups.

Isozyme markers

Twenty-four segregating markers from thirteen isozyme systems were scored in total. Seventeen isozyme markers from twelve isozyme systems were mapped. *PRX-2* and *PRX-3* showed no recombinants. Ten of the isozyme markers were mapped near the distal ends of the linkage groups (Fig. 1). With the exception of *PRX-2* and *PRX-3*, markers from one system were always located on different linkage groups.

AFLP markers

Nine fragments showed segregation and were scorable: six 'Prima' markers, two 'Fiesta' markers and one marker present in both parents. Seven more fragments segregated but could not be scored reliably. An extra 60 bands were clear but did not segregate. No significant LOD scores were obtained for 'Prima' marker E35/M47/P3. A second 'Prima' marker (E35/M47/P6) showed distorted segregation and, although the marker could be assigned to L05, was discarded since no recombination frequencies smaller than 0.19 were obtained with any of the other markers. One of the 'Fiesta' markers was assigned to linkage group L05 but was discarded at the mapping stage due to inconsistencies in the estimated recombination frequencies. Two of the 'Prima' markers were linked in repulsion and showed no recombinants, thus behaving as alleles. (E35/M47/P4 and E35/M47/P5).

SCAR marker

The BC226 marker was scored as the presence or absence of the a^2 allele segregating from the 'Fiesta' parent (Cheng et al. 1996). The other 'Fiesta' allele was A^2, as was the only detectable allele in the 'Prima' parent. As the A^2 allele was detectable in all screened progeny, it is reasonable to assume that 'Prima' is homozygous at this locus. The segregating allele is thought to be linked to the absence of red anthocyanin pigmentation but is overridden in all progeny by the dominant presence of the pigment linked to the A^2 allele. This is consistent with red colouration of all the fruit in this progeny. BC226 was mapped to linkage group L09-Fi.

Segregation types

The segregation types of mapped markers is shown (Table 1). The numbers of markers indicate a higher level of heterozygosity for 'Prima' than for 'Fiesta', although this may be somewhat biased for the RAPD markers because of specific searches for markers in coupling phase with *Vf*. RFLP markers were preferably selected if they were heterozygous in both parents.

Single-locus analysis

Thirty-three of the marker loci displayed distorted segregation ($P < 0.05$; chi-square test) and are indicated

Table 1 Segregation types of markers mapped in the 'Prima' × 'Fiesta' progeny

	$ab \times cd$	$ab \times ac$	$ab \times ab$	$ab \times aa$	$aa \times ab$	Total
RFLP	14	25	18	37	30	124
RAPD	0	0	0	80	53	133
SSR	4	5	0	0	1	10
Isozyme	1	0	0	6	10	17
AFLP	0	0	0	3	1	4
CAPS-RFLP	0	0	0	1	0	1
SCAR	0	0	0	0	1	1
Total	19	30	18	127	96	290

with asterisks in Fig. 1. The 'Fiesta' and 'Prima' alleles showed distorted segregation in 21 and 10 cases, respectively, while in two cases $ab \times ab$-type markers showed segregation distortion, so that for these the parent generating the distortion is unknown. The distorted markers were found mainly in two regions on linkage groups L02 and L10. These were also the regions with the most severely distorted segregation ratios. As can be seen in Fig. 1, for example for linkage group L02-Fi, markers with a high significance value for the chi-square test occur in close linkage with markers with lower significance values. This is due to differences in segregation types and in the numbers of individuals genotyped. For instance, MC116b and MC029a on L02 both have an $ab \times ab$ segregation type and are tested against the 1 : 2 : 1 ratio, whereas the other markers are tested against the 1 : 1 ratio. For $aa \times ab$ markers, a higher frequency of one of the 'Fiesta' alleles is directly observed in the higher abundance of either the *aa* or the *ab* genotype, whereas for $ab \times ab$ markers the distortion is observed only in overabundance of one homozygote at the cost of the other while the frequency of the heterozygote is as expected.

Map construction

The initial grouping of markers, based on a JoinMap LOD score of 4.0, resulted in 16 linkage groups, all groups combining markers heterozygous in the female parent, the male parent and both parents. One large linkage group consisted of two subgroups (L01 and L12) held together by a single marker pair. Further investigation demonstrated that only 25 individuals had been scored for both markers, that the scoring of one of these markers was difficult and that the JoinMap LOD score of 4.2 deviated strongly from the traditional LOD score for linkage, which was 1.3. No other markers suggested linkage of the two groups. The problematic marker was discarded, and the two groups were separated so that seventeen linkage groups remained. These were divided into separate linkage groups for each of the parents. Sixty-seven markers (57 RFLPs, nine microsatellite markers and one isozyme marker) were heterozygous in both parents (Table 1) and allowed the identification of homologous pairs of linkage groups of the 'Prima' and 'Fiesta' parents. The seventeen pairs of linkage groups have been numbered from L01 to L17 (Fig. 1).

An integrated map was also calculated (not shown). In some instances there were differences in the estimated marker order between the individual parental maps and/or the integrated map. For instance, for linkage group L02 the orders of markers heterozygous in 'Prima' and 'Fiesta' were identical. However, the order of the 'Fiesta' markers in the integrated map deviated from the L02-Fi order due to the differences in recombination frequency estimates in 'Prima' and 'Fiesta' for markers heterozygous in both. For linkage group L06-Pr the initial analysis resulted in a marker order with MC023 and MC034 swapped in comparison with the 'Fiesta' order. Using all markers from both parents resulted in the 'Fiesta' order. Using a JoinMap option to enforce a fixed order, the 'Fiesta' marker order was enforced for the 'Prima' linkage group. This resulted in only a slightly smaller likelihood. For L04 the marker order of MC013, NZ05g8 and MC019 was estimated differently in 'Prima' and 'Fiesta'. Enforcing the 'Fiesta' order for the 'Prima' map resulted in unresolvable negative marker distances, since many linked markers confirmed the originally estimated order. Enforcing the 'Prima' order on the 'Fiesta' map resulted in a solution which was slightly suboptimal but acceptable (mean chi-square less than 2.0), so that this order was chosen. For L01 and L11 the initially estimated marker order for the integrated map differed from both the 'Prima' and the 'Fiesta' order, but enforcing these orders for the integrated map resulted in a better solution.

Comparison of recombination frequencies

The level of recombination was compared between 'Prima' and 'Fiesta' for non-overlapping segments of the linkage groups. For this, segments were used that are flanked by one or two $ab \times cd$- or $ab \times ac$-type markers and possibly on one side by an $ab \times ab$ type marker. To test whether recombination was smaller in 'Prima' than in 'Fiesta', we applied the signed-rank test of Wilcoxon (e.g. Lehmann 1975). For eight out of 48 segments the recombination frequencies were identical for both parents, so that the test was applied to 40 segments. In twelve cases the 'Fiesta' estimate was smaller than the 'Prima' estimate; for the remaining 28 segments the 'Prima' estimate was smaller. This indicated that, in general, recombination was smaller in 'Prima' than in 'Fiesta' ($P < 0.05$). However, there were some differences between linkage groups: on linkage groups L12 and L17 some recombination frequencies were smaller for 'Fiesta'. On linkage group L10 the 'Prima' recombination frequencies were smaller for the top (where the segregation was distorted for the 'Prima' alleles), whereas 'Fiesta' recombination frequencies were smaller for the bottom of this linkage group (where the segregation was distorted for the 'Fiesta' alleles). On this linkage group some differences in the recombination frequency estimates were quite large. For example, the direct recombination frequency of 2E11 and MC227 was 0.20 for the 'Prima' alleles, whereas the recombination frequency in 'Fiesta' was larger than 0.5. However, in between these two markers there are several other markers confirming linkage.

Monogenic traits

The *Vf* gene for scab resistance was mapped to the distal end of linkage group L01-Pr as described in King et al. (1998). The gene Sd_1 for resistance to (two biotypes of) the rosy leaf curling aphid (*D. devecta*) was mapped to the distal end of linkage group L07-Fi closely linked to three RFLP markers (Roche et al. 1997a).

Assuming a genetic model $Mama \times Mama$ for the fruit pH data, we mapped the *Ma* gene for fruit acidity to the distal end of linkage group L16.

The self-incompatibility locus was mapped closely linked to *AAT-1* on linkage group L17. The *S* combination S_3S_5 for 'Fiesta' confirmed results by Batlle et al. (1995). The red fruit gene *Rf*, which did not segregate in this cross, can be assigned to linkage group L09 through the linked SCAR BC226. The gene for columnar tree habit, *Co*, can be assigned to linkage group L10 through the linked SSR marker USA-SSR11. The Pl_w gene from the ornamental apple 'White Angel' for resistance to powdery mildew (*Podosphaera leucotricha*) can be assigned to linkage group L08 on the basis of previously detected linkage to *LAP-2* (Manganaris and Alston 1992).

Duplicate markers

Thirty-seven of the probes used in this study generated markers for two or more loci. Van Heusden et al. showed that a majority of cDNA clones detects two loci in apple (personal communication). It was not only observed that certain clones detected two loci on different linkage groups but, moreover, that in six instances a set of RFLP loci linked on one linkage group also showed a set of corresponding RFLP loci, from the same clones, linked on another linkage group (Fig. 1). On linkage groups L02 and L07 markers of clones MC029, MC064 and MC116 are linked. Markers MC019 and MC034 are linked on L04 and L06, MC040 and 7Bg on L06 and L14, while MC023 has markers on L04, L06 and L14. MC041, MC001, LY05 and MC221 are linked on L13 and L16. Eight markers linked on linkage group L05 have corresponding markers on linkage group L10. Markers on linkage group L14 have corresponding markers both on L06 and L12. The marker orders are not always identical; for example, the order of seven markers is identical on L05 and L10, but LY29 is at different positions on these linkage groups. On L02 and L07 the order of MC029 and MC064 is reversed with respect to MC116, but here it should be taken into account that there are hardly any recombinants between the two markers so that the likelihood of a reverse marker order on either of the two linkage groups is only slightly smaller. In most cases of linked duplicate markers the map distances are similar.

Map length and density

The total map length for 'Prima' is 842 cM, for 'Fiesta' 984 cM. Considering the numbers of markers in 'Prima' (194) and 'Fiesta' (163) this corresponds to a mean density of one marker every 4.3 cM for the 'Prima' map and one every 6.0 cM for the 'Fiesta' map. However, markers are not uniformly distributed over the maps. Comparison of the map lengths of linkage groups of the two parents and the positions of markers present in both parents suggests that part of the ends of L01-Fi (bottom), L03-Pr (top), L10-Pr (bottom) L11-Pr (bottom), L15-Fi (bottom) and L17-Pr (top) are missing. In addition, there are widely spaced marker intervals (> 20 cM) at L02-Pr/Fi, L03-Pr/Fi, L04-Fi, L05-Fi, L08-Fi, L09-Pr/Fi, L16-Fi and L17-Pr/Fi. An attempt to provide (RAPD) markers for two of these intervals (L05 and L16), using Bulked Segregant Analysis based on the markers flanking the intervals, failed. No polymorphisms between the bulks were observed in 165 clear bands from 36 primers for L05 and 309 bands from 62 primers for L16.

Discussion

Marker detection

Large numbers of segregating markers could be found with all marker types. RAPD primers yielded on average 2.0 scorable and segregating markers. About half of the isozyme markers tested yielded a marker which segregated in this progeny. From 133 'MC' RFLP clones only five did not generate a polymorphism with any of the six restriction enzymes (Van Heusden et al. personal communication). Where the RFLPs are concerned, some choice of segregation type was possible, depending on the restriction enzyme used, and where possible the probe/enzyme combination which yielded the most informative type(s) of segregation ($ab \times cd$ or $ab \times ac$) in the progeny was chosen. Nine of the ten microsatellite markers used also displayed the most informative segregation types. This illustrates that finding markers for mapping studies in apple is not difficult, as was also observed by Hemmat et al. (1994) and Conner et al. (1997). Chaparro et al. (1994) reported much lower levels of polymorphism (for RAPD markers) in peach, which is autogamous. Rajapakse et al. (1995) found 50% of the primers to produce polymorphic fragments in peach. Polymorphism in almond, which is only preferentially autogamous, was found to be much higher than in peach, but not so high as in apple (Viruel et al. 1995).

Map length and density

This linkage map consists of seventeen pairs of homologous linkage groups from 'Prima' and 'Fiesta' and

may well represent the seventeen pairs of chromosomes, considering the progeny sze and the result that most markers which were genotyped on at least 50 plants could be placed on this linkage map. Markers which nonetheless could not be mapped usually displayed a complex banding pattern and/or skewed segregation ratios. In those cases, multiple bands may be overlapping. For at least one of the mapped markers (MC038) we also identified bands with similar sizes from two different loci.

The average map size per linkage group, 50 cM for 'Prima', 58 cM for 'Fiesta', is considerably smaller than the 100–150 cM which is commonly found in agricultural crops. Other tree species, such as *Eucalyptus*, *Theobroma cacao* and *Pinus radiata* also have considerably larger linkage groups (Grattapaglia and Sederoff, 1994; Byrne et al. 1995; Lanaud et al. 1995; Devey et al. 1996). As indicated, some parts of linkage groups are probably missing. Discarded markers may also partly explain smaller linkage groups but not to the extent as is observed here. The small map is in agreement with the 950 cM for the crabapple 'White Angel' (Hemmat et al. 1992) and with map sizes in the range of 692 to 898 cM for the single-parent maps in Conner et al. (1997). Moreover, it should be noted that the small linkage groups of apple are not unique within the Rosaceae: comparable sizes were observed and discussed for peach and almond, which yielded a total map length of 400 cM for eight linkage groups (Chaparro et al. 1994; Viruel et al. 1995). Dickson et al. (1992) reported that the nuclear DNA content of the Rosaceae is low among angiosperms, and although DNA content per map unit is known to vary widely across plant species (e.g. Nodari et al. 1993), the consistency between *Malus* and *Prunus* may indicate that in Rosaceae both DNA content and map size per haploid genome are small. However, a general conclusion cannot be inferred until more linkage maps of Rosaceous species have become available.

Integration of parental maps

The availability of codominant markers allowed not only the identification of homologous linkage groups but also the integration of both parental maps. For most linkage groups the integrated map was consistent with the marker orders in the individual parental linkage groups, although some minor differences with respect to marker orders were observed, usually with small differences in the likelihoods of these marker orders. However, where large differences in the recombination frequencies in both parents were observed, such as for L02, the integration of both parental maps caused marker orders to change. The integrated map combines markers segregating in one or the other parent with those segregating in both parents. For marker pairs heterozygous in both parents the combined recombination frequency estimate is an average over the recombination frequencies in the male and the female meioses. This combined estimate may differ from the single-parent estimates and thereby cause a changed marker order in the integrated map in comparison with the single-parent map. Since differences in the estimated distances of both maps may reflect real differences in the recombination frequencies of both parents, these can best be presented separately. Both parental maps can be used separately to investigate quantitative trait loci (QTLs) segregating from a single parent. However, if QTLs may be present in both parents and for studying the different allelic combinations at QTLs, it is better to use the integrated map with an all-marker mapping approach (Knott and Haley 1992; Maliepaard and Van Ooijen 1994).

Duplicate markers

The origin of the Maloideae subfamily of the Rosaceae has been subject to some debate (e.g. Chevreau et al. 1985; Chevreau and Laurens 1987; Phipps et al. 1991; Morgan et al. 1994). The basic chromosome number, $x = 17$, suggests a polyploid origin since other Rosaceae have $x = 7$, 8 or 9. Autopolyploidy was suggested by Darlington and Moffet (1930), but more recent studies have shown only bivalent pairing of the chromosomes in meiosis (Lespinasse 1973) and monogenic or bigenic disomic inheritance of isozymes, which is in agreement with diploid behaviour of single or duplicated genes, and thereby supports an amphidiploid origin (Chevreau et al. 1985; Chevreau and Laurens 1987; Weeden and Lamb 1987). Phipps et al. (1991) discussed an allopolyploid origin with Amygdaloideae ($x = 8$) and Spiraeoideae ($x = 9$) as ancestors. Morgan et al. (1994) provided evidence that at least one progenitor was a Spiraeoid and suggested that $x = 17$ resulted from aneuploid reduction from $x = 18$ either by autopolyploidy or allopolyploidy. Van Heusden et al. (personal communication) showed that a majority of cDNA clones corresponded to two loci with a disomic inheritance.

The present results demonstrate not only that duplicate RFLP markers are abundant in the apple genome, but also that in a number of cases linked sequences on one linkage group, as detected by RFLP clones, can also be found at another linkage group. Considering the proposed polyploid origin of apple this is not unexpected. However, the results suggest differences in the amount of homology across linkage groups: L05 and L10, for example, have eight markers in common, suggesting sequence homology across large portions of these linkage groups. Other pairs of linkage groups do not show such a high amount of homology. L04 and L06, for example, share a pair of markers from two clones, but other markers on L06 have counterparts on L14 and L05, while one marker has counterparts on both L04 and L14. Also, markers

on L14 do not only have counterparts on L06 but also on L12. These results suggest that large chromosomal regions are conserved between and possibly also within the basic genomes underlying the apple genome. This is consistent with the proposed amphidiploid origin of apple and suggests that the ancestors of the original hybrid were closely related. It is worth noting that Viruel et al. (1995) reported that a majority of cDNA clones in *Prunus* (x = 8) identified single loci and that the (genomic and cDNA) clones that detected two loci did not provide evidence for the existence of duplicated chromosomes or of large duplicated chromosomal parts. The SSR markers used in this study did not detect multiple loci across the apple genome, but Guilford et al. (1997) observed that approximately 25% of their (GA)-repeat markers showed complex banding patterns consistent with two loci and they verified independent bigenic inheritance for one of these.

Mapping of monogenic traits and QTLs

The apple map presented in this study combines different marker types and has, overall, a high marker density, although there still are a few regions with wide marker intervals. Map positions were provided for *Vf* for scab resistance, Sd_1 for resistance to rosy leaf curling aphid, *Ma* for the presence of malic acid and the self-incompatibility locus *S*. This map can be used for QTL mapping purposes and, considering the large number of codominant markers, especially RFLPs, can probably be transported to other progenies. A higher number of microsatellites per linkage group would greatly facilitate this. At present, microsatellite markers for apple are being evaluated by the different mapping groups (Guilford et al. 1997; C. Ryder et al. personal communication; N. F. Weeden, personal communication).

Comparison with other maps

There are some markers in common between this map and the maps published by Hemmat et al. (1994) for 'Rome Beauty' (RB) and 'White Angel' (WA) and by Conner et al. (1997) for 'Wijcik McIntosh' (WM), and the 'Prima' × 'Spartan' selections NY-67 and NY-58. Corresponding markers identifying probably homologous linkage groups are indicated in Table 2.

It can be concluded that our L01 corresponds to USA-L08, L02 to USA-L14, L03 to USA-RB5/WA8 = USAnewL10 (RB-5 and WA-8 are now considered to be homologous and are coded here as RB-10new and WA-10new; RB-10 was found to be homologous to WA-5; N. F. Weeden, personal communication), L07 to USA-L09, L08 to USA-L07, L09 to USA-L03, L10 to USA-L06, L14 to USA-L02, L17 to USA-L01 (and maybe also to USA-L17).

In addition, RFLP clones pAP79 and pAP260 were used in both studies. One marker from clone pAP79 was mapped to linkage group L02 and may correspond to pAP79 on linkage group WA-13, although *ME-1* suggests that our linkage group L02 corresponds to RB-14. pAP260 generated two markers, one on linkage group L11 and one on linkage group L16. One of these may correspond to pAP260 on WA-15 of Hemmat et al. (1994). The *Co* gene was mapped to linkage group 10 in Conner et al. (1997), corresponding to our linkage group L10. This map position of the *Co* gene was confirmed in a 'Fiesta' × 'SA572/2' progeny which also segregates for the columnar tree habit.

Our map positions of *Vf* and *Ma* on two different linkage groups, L01 and L16, respectively, are in contrast with those of Conner et al. (1997), who placed *Vf* and *Ma* on a single linkage group. However, this was based on the inference of homology of a small WM linkage group and a NY-67 linkage group through only

Table 2 Markers in common between our map and the maps of Hemmat et al. (1994) and Conner et al. (1997) (microsatellite positions from personal communications)

Marker	Hemmat or Conner designation	Our linkage group	Hemmat or Conner linkage group[a]
PGM-1	*Pgm-p1*	L01	RB-8
TPI-5	*Tpi-c2*	L01	RB-8
ME-1	*Me*	L02	RB-14
PRX-2	*Prx-A*	L03	RB-5old/RB-10new[b]
MS14h03		L03	WA-8old/WA-10new[b]
PGM-2	*Pgm-2*	L07	WA-9
AAT-2	*Aat-p*	L08	WA-7
NZ04h11		L09	3[b]
BC226	*BC226*	L09	3
EST-1	*Est-1*	L10	WA/RB-6
MS02a01		L10	6[b]
USA-SSR11	*Co SSR*	L10	6[b] (10 in Conner et al.)
PGD-1	*Pgd-p*	L14	WA-2
MS01a05		L14	2[b]
AAT-1	*Aat-c*	L17	WA-1
FDH-1	*Fdh*	L17	WA-17

[a] WA, White Angel linkage group; RB, Rome Beauty linkage group
[b] Personal communication from Dr. N. F. Weeden

a single allelic bridge. Such a pairing should be viewed with caution, as was also pointed out by Conner et al. (1997).

Prospects for comparative mapping

This map combining large numbers of RFLP, SSR, RAPD and isozyme markers offers possibilities for comparative mapping with other important Rosaceous genera, such as *Pyrus*, *Prunus*, *Rosa* and *Fragaria*. For isozyme markers, a first comparison between apple and pear has been made by Chevreau et al. (1997). At present markers are being exchanged among *Prunus* mapping groups and the European, New Zealand and USA mapping groups of apple to establish a basis for comparative genomic studies.

Prospects for marker-assisted selection

Molecular markers for important characters in apple could greatly reduce the amount of space and time required for breeding. In an apple breeding programme, marker-assisted selection should be applied before seedlings are planted in the field. Preferably, multi-allelic reproducible markers should be used which require minimal amounts of DNA and a minimum of DNA isolation and purification steps, so that large numbers of plants can be screened in a short period of time. Considering these aspects, SCARs are the markers of choice at present and these have been developed for a number of traits. For *Vf*-based scab resistance, SCARs are available. Although seedlings carrying *Vf* can normally be selected accurately with traditional screening methods, markers for *Vf* resistance may be highly efficient for pyramiding monogenic resistance genes or for combining *Vf* with genes for partial resistance. These markers may also be useful for selecting for *Vf* resistance in the absence of the pathogen, e.g. if no inoculum is present in certain years or at some location, or in the presence of races which have overcome *Vf*, such as race 6 (Parisi et al. 1993). Furthermore, these markers can be useful for eliminating the portion of *Malus floribunda* genome around the resistance gene and for identifying *Vf* homozygotes.

In contrast with scab, resistance to rosy leaf curling aphid can more readily be scored with markers than in field or greenhouse tests, but the economic importance of the pest is relatively small. Markers for fruit characters would have a high efficiency since these phenotypic traits can be evaluated only after five or six years, once the tree has passed through it's juvenile phase. For instance, a marker specific for the presence of *Ma* could save considerable costs in apple breeding programmes where many crosses involve two heterozygous parents. In such crosses an expected quarter of the progeny yields tasteless fruits without malic acid. A marker for *Ma* would allow selection at an early seedling stage before field planting and could save up to six years of tree care. Markers can also be used for other purposes than selection: Janssens et al. (1995) suggested that the *S*-allele-specific markers may be helpful in the choice of fully compatible pollinators to provide optimal fruit set for new cultivars.

This linkage map provides an important tool not only for the detection of simple major genes but also for more complex QTLs and for providing breeders and researchers with markers for these genes. The map may also be helpful in studying the interaction of genes or establishing evidence of allelism. In the future, this map may provide an essential tool for map-based cloning techniques. Improvement of popular apple cultivars with a single gene, impossible with conventional breeding techniques, is likely to become possible once important genes, such as scab resistance genes, have been cloned.

Ideally a reference linkage map should contain at least a backbone of codominant markers, such as RFLPs or SSRs, which are reproducible, can be transported to another progeny and which can then be supplemented with RAPD or AFLP markers to saturate the more interesting regions of the genome. The present map fulfills these requirements and is therefore an ideal core map for apple genetic research.

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