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AFLP maps of *Petunia hybrida*: building maps when markers cluster

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Abstract AFLP mapping in *Petunia hybrida* was undertaken with the intention of building a high-density genetic map suitable for applications such as map-based gene cloning. In total five maps were constructed from two mapping populations, with placement of more than 800 markers. Despite the large number of markers the resulting map is roughly ten-fold smaller than those of other plant species, including the closely related tomato. Low levels of recombination are reflected in clusters of tightly linked markers, both AFLPs and RFLPs, in all the maps. Clustering patterns vary between mapping populations, however, such that loci tightly linked in one population may be separable in another. Combined with earlier reports of aberrant meiotic pairing and recombination, our results suggest that, for species like petunia, map-based cloning may be more complex than in model species such as arabidopsis and tomato.

Keywords AFLP · *Petunia hybrida* · Genetic map · Marker clusters · Recombination

Introduction

Petunia hybrida Vilm is a horticulturally important dicot of the solanaceous family with a moderate genome size (1.2×10^6 kb, Arumuganathan and Earle 1991) and just seven chromosomes in the haploid complement. It features a rich history of genetic and cytological investigation. Crosses are straightforward, capable of producing hundreds of seeds per cross. Although used primarily as an annual bedding plant, it grows as a perennial in warm climates and can be maintained indefinitely in the greenhouse. Transformation is almost as straightforward as with tobacco, making petunia an attractive subject for transgenic studies as well (Wallroth et al. 1986).

The origin of *P. hybrida* is unknown, but there is general agreement that it was produced by crossing a white-flowered species (probably *Petunia axillaris* or perhaps *parodii*) with a pigmented species (*Petunia violacea*, *integrifolia or inflata*) (Wijsman 1982, 1983; Sink 1984). The genetic diversity associated with its hybrid origin has proved useful in the identification of many traits of interest in plant breeding, including those associated with self-incompatibility, pigmentation and floral development (see, e.g. Martin and Gerats 1993; Colombo et al. 1997; Mol et al. 1998; Robbins et al. 2000). Over the past decade both forward (Souer et al. 1995; Van den Broeck et al. 1998) and reverse (Koes et al. 1995; Maes et al. 1999, 2001) genetic approaches have been developed and applied successfully to recover mutant alleles and characterise mutant phenotypes in petunia.

A potentially useful forward approach requiring only a mutant phenotype and a robust genetic map is that of map-based cloning. The availability of lines with significant DNA sequence variation permits the construction of high-density linkage maps, used to recover genes by virtue of their chromosomal positions relative to other markers. With petunia, as with many other species, construction of a high-density genetic map was thus an obvious step toward map-based cloning of desired genes.

The classical genetic map of petunia consists of approximately 120 phenotypic markers (Gerats et al. 1993), for the most part assigned to chromosomes or large chromosomal regions. RFLP mapping was used to map chalcone synthase genes (Koes et al. 1987) and a few years later to map members of the actin gene family (McLean et al. 1990). Complementary DNA and genom-

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ic probes were subsequently used to add 28 additional loci to a combined RFLP-phenotypic map (Strommer et al. 2000). Meanwhile Peltier and coworkers (1994) constructed a fused RAPD-phenotypic map with 35 RAPD markers, demonstrating the existence of the same markers in potential ancestors of *P. hybrida*. Cytological mapping, based on deletions and partial trisomics, allowed the assignment of 14 phenotypic markers to chromosomal segments (Maizonnier et al. 1986), creating the basis for a physical map.

PCR-based amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995) has been used in the preparation of regional and high-density maps to facilitate breeding or map-based cloning in a number of plant species (e.g. Cnops et al. 1996; Alonso-Blanco et al. 1998; Castiglioni et al. 1998; Simons et al. 1998; Bert et al. 1999; Castiglioni et al. 1999; Debener and Mattiesch 1999; Vuylsteke et al. 1999). Gerats and coworkers (1995) previously introduced AFLP mapping as a fast and reliable method for genetic mapping in petunia. To begin construction of a high-density physical-genetic map suitable for map-based cloning, we therefore employed AFLP analysis, using the same DNA samples from the three populations on which the earlier RFLP maps (McLean et al. 1990; Strommer et al. 2000) were based.

The RFLP map of Strommer et al. (2000) measured 368 centi-Morgans, with evidence for less than the expected one crossover per meiotic bivalent. Our expectation was that the addition of a large number of AFLP markers would fill in gaps and expand the genetic map. Here we describe a series of maps based on recombination in two different F1 hybrids. Locus order, though not genetic distance, is conserved. The maps are very short, with tightly clustered markers; the clustering patterns, however, are chromosome- and cultivar-specific. Our results are considered in relation to other AFLP maps and to the origin and cytology of *P. hybrida*.

Materials and methods

Plant material

The populations of *P. hybrida* used for AFLP mapping are summarized in Table 1. The inbred lines V23 and R51 carry different alleles for easily scored phenotypic markers on each of the seven chromosomes (Wallroth et al. 1986). *P. hybrida* var. Mitchell, is a doubled-haploid derived from anther culture of a *P. hybrida–P. axillaris* hybrid (Mitchell et al. 1980). Mapping populations derived from these lines, designated V23, R51 and M, as described in Table 1, are the same as those used to generate the earlier maps of McLean et al. (1990) and Strommer et al. (2000).

The W137 line is characterized by the presence of many phenotypic markers associated with the activity of an endogenous transposable element system (Doodeman et al. 1984). A V26 × W137 hybrid was backcrossed to the W137 parent to produce a mapping population unrelated to V23 and R51, designated W137.

In total, the mapping populations consisted of 48 individuals for each of the two VR backcrosses (designated V23 and R51), 79 for the Mitchell cross (M), and 61 for the W137 backcross (W137). The designation VRM refers to the combined VR-based populations (V23, R51 and M), comprising 175 individuals.

Table 1 Mapping populations used in this study

Designation	Female parent	Male parent(s)
V23	V23 × R51 hybrid	V23
R51	V23 × R51 hybrid	R51
M	V23 × R51 hybrid	Mitchell
VRM	V23 × R51 hybrid	V23, R51 and Mitchell
W137	V26 × W137 hybrid	W137

Table 2 Primer combinations used in AFLP analysis

Digest and selective nucleotides		
*Eco*RI	*Mse*I	Designation
AAC	AGC	E32M40
ACA	AAC	E35M32
	ACA	E35M35
	ACC	E35M36
	ACG	E35M37
	AGA	E35M39
	AGG	E35M41
	AGT	E35M42
ACC	CAT	E36M50
AGA	ACA	E39M35
	ACC	E39M36
	ATC	E39M44
	CTT	E39M62
CAG	AAC	E49M32
	ACA	E49M35
	ACC	E49M36
	ACG	E49M37
	ACT	E49M38
	AGA	E49M39
	AGC	E49M40
	AGG	E49M41
	AGT	E49M42
	ATC	E49M44
*Sac*I	*Mse*I	
ACA	AGC	C35M40

AFLP analysis

Preparation of DNA from the V23, R51 and M populations has been described (McLean et al. 1990; Strommer et al. 2000). DNA from the W137 population was prepared from young leaves as described by Dellaporta et al. (1983), but without the final isopropanol precipitation. Proteins were extracted by addition of an equal volume of high-salt CTAB (2% w/v cetyltrimethylammonium bromide, Sigma Corp, St. Louis, Mo.; 200 mM Tris.Cl, pH 7.5; 2 M NaCl; 50 mM EDTA), after which DNA was precipitated by the addition of 2 vol of ethanol. Pelleted DNA was resuspended in 100 µl $^{0.1}$TE (10 mM Tris.Cl, 0.1 mM EDTA, pH 8.0) prior to restriction endonuclease digestion.

The AFLP methodology was that described by Vos et al. (1995), using cleavage with *Eco*RI (or in one case *Sac*I, both from Pharmacia, Uppsala, Sweden) and *Mse*I (New England Biolabs, Beverly, Mass.). Adaptors and primers were obtained from Genset (Paris, France). Restricted amplification relied on the addition of three selective nucleotides. Nomenclature used for fragment identification is summarized in Table 2. All amplification reactions were performed in a Perkin Elmer 9600 thermocycler (Perkin Elmer Corp., Norwalk, Conn.). DNA fragments were separated in a Sequi-Gen GT gel electrophoresis apparatus (Biorad, Hercules,

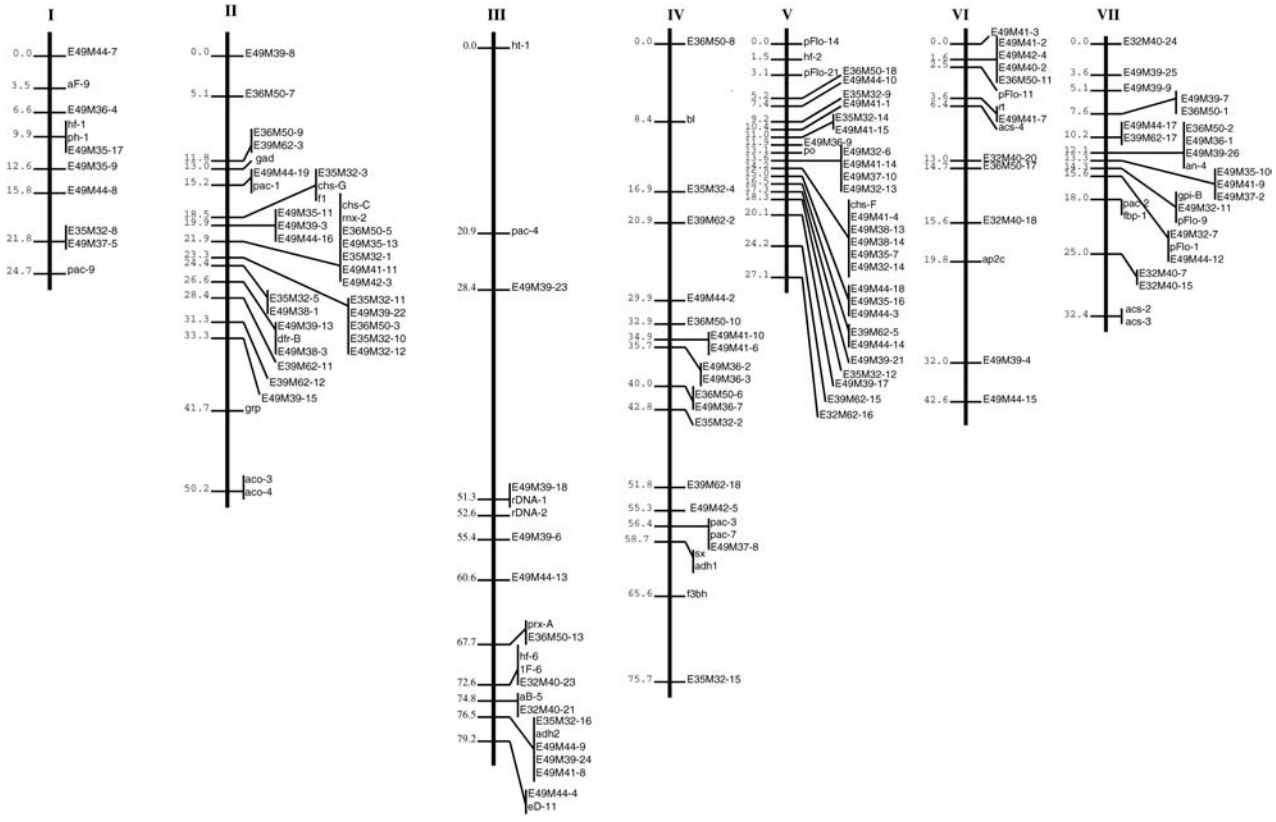

Fig. 1 Genetic map of *P. hybrida* based on combined AFLP, RFLP and phenotypic marker analysis of V23-R51 hybrids, measured in progeny from V23 and R51 backcrosses and an outcross to *P. hybrida* var. Mitchell. Orientations of the linkage groups match chromosomal orientations used by Maizonnier et al. (1986). Cumulative distances in centi-Morgans are given on the left and markers on the right; markers joined by a *bar* exhibited no recombination. *Brackets* to the left of chromosomes II and V indicate major clusters of loci

Calif.), and gels dried in a Heto Dry GD apparatus (Heto Lab Equipment, Denmark). Amplified fragments were visualized by means of autoradiography on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Five sets of AFLP data were generated, for the V23, R51, M and W137 populations individually and for V23, R51 and M samples amplified and analyzed as a unit.

Southern hybridizations

For hybridization analysis of AFLP fragments, the region of the acrylamide gel corresponding to a specific band was excised using the autoradiogram as a guide. DNA was eluted overnight in 100 µl $^{0.1}$TE at 4 °C. Acrylamide and the filter paper on which it had been dried was pelleted, and the DNA recovered by ethanol precipitation. This DNA was dissolved in 100 µl $^{0.1}$TE. Three microliters were diluted to 50 µl in PCR buffer (Perkin Elmer) and subjected to 25 cycles of PCR using one unit of *Taq* polymerase (Perkin Elmer, Norwalk, Conn.), with 30 s @ 94 °C, 1 min @ 60 °C, and 1 min @ 70 °C. Aliquots were subjected to electrophoresis in 1.5% agarose, then transferred by capillary action to Hybond-N membranes as recommended by the supplier (Amersham, Buckinghamshire, UK). Another aliquot of eluted DNA was labeled by random priming with [α-^{32}P] dCTP (Amersham) using a Megaprime DNA labeling kit (Amersham). Hybridizations were performed overnight in 6 × SSC, 5 × Denhardt's, 0.05% sodium pyrophosphate and 20 µg/ml of calf thymus DNA at 60 °C. Filters were washed in 2 × SSC, 0.1% SDS at 60 °C, followed with washing in 0.1 × SSC, 0.1% SDS at the same temperature for approximately 15-min each. Bands were revealed by autoradiography. Before rehybridization, boiling 0.1% SDS was poured over the blot, which was then shaken for several minutes to remove bound probe.

Linkage analysis

Pre-existing RFLP data from McLean et al. (1990) and Strommer et al. (2000), together with those obtained by Maes for *apetala-2* (1998) and Sauer and Gerats for *pFlo* (data unpublished), were combined with newly generated AFLP data. These were analyzed using Joinmap version 2.0 (Stam 1993; Stam and van Ooijen 1995). The mapping populations were defined as BC1 (first generation backcross). A LOD of five allowed grouping of markers into linkage groups, and the following parameters were used to order markers: REC 0.499; LOD 0.001; jump 5; ripple 3 and triplet 7. Apparent single-locus double-crossovers were either validated or corrected after re-examination of gel images. Information on specific markers, including gel images, will be made available on request.

Results

Recombination in crosses involving V23 and R51

The map obtained by combining analysis of the three VR-based populations (VRM), derived from a total of 13,678 data points, is presented in Fig. 1. With minor exception there is good agreement with the RFLP map generated with the same DNA samples (Strommer et al. 2000). The exceptions involve a repositioning of the two

Table 3 Lengths of linkage groups among AFLP mapping populations in relation to the number of markers. # = number of markers; cM = length of linkage group in centimorgans

Chromosome	I		II		III		IV		V		VI		VII		Total	
	#	cM	#	cM	#	cM	#	cM	#	cM	#	cM	#	cM	#	cM
VRM	11	24.7	36	50.2	22	79.2	22	75.7	31	27.1	15	42.6	26	32.4	163	331.9
V23	23	25.5	38	43.5	33	57.2	37	21.8	32	25.1	14	24.8	84	43.8	236	241.7
R51	21	23.9	30	14.3	47	68.6	40	43.1	40	21.9	23	42.2	46	6.9	247	220.9
M	9	51.8	22	52.0	10	51.4	16	40.8	21	17.2	8	12.7	13	48.3	97	274.2
W137	51	44.6	52	14.7	23	42.9	18	18.6	14	79.5	15	12.8	55	47.8	228	260.9

ribosomal DNA loci closer to each other on chromosome III, and the inversion of a cluster at one end of chromosome IV. Disappointingly, with the addition of 127 AFLP markers and nine additional RFLP loci, map length is shortened (from 368 to 332 cM). The new map accentuates the previously demonstrated tendency toward locus clustering: on chromosome II, for example, 28 out of 36 markers lie within 17 cM of one another; and on chromosome V, 26 of 31 markers assigned to the chromosome cover less than 15 cM.

The V23, R51 and M mapping populations all featured a female V23 × R51 hybrid parent; for VRM map construction AFLP products representing the three populations were generated simultaneously and products were separated side-by-side on acrylamide gels to ensure correct band identification. The perceived advantage of merging these populations was the resulting maximization of population size (175 individuals compared with 48, 48 and 79, respectively, for the V23, R51 and M populations). However, the combining of populations created a complex data set for JOINMAP: by AFLP, segregation data for a specific marker locus can be obtained from one set of backcross progeny but not the other. Thus segregation data for a single marker could be obtained from either V23 and M, or R51 and M, populations but never from all three. This resulted in basically two separate data sets, bridged by limited RFLP data. Furthermore, by limiting data points to polymorphisms revealed in two populations (either V23 and M, or R51 and M), polymorphisms identified within a single population were unusable. The three populations considered independently, while smaller, therefore contribute unique and informative data.

The generation and analysis of AFLP data for individual populations allowed us to construct independent maps of the V, R and M populations. Relevant characteristics of these maps, in comparison to the VRM map, are summarized in Table 3, which includes the number of loci mapped and genetic length of each chromosome. In all cases the genetic map length decreased by roughly a third, with no correlation between the number of loci mapping to a chromosome and its length, in comparison to that on the combined VRM map.

The (V26 × W137) × W137 testcross

The possibility remained that our results reflected some oddity associated with recombination in V23 × R51 hybrids specifically, but atypical for *P. hybrida* as a species. This hypothesis was tested by creation of a new AFLP map based on a totally unrelated pair of lines, V26 and W137. This map, constructed from 12,879 data points representing 61 individuals, is presented in Fig. 2. A summary of chromosome lengths is included in Table 3. The length of the W137 map, at 261 cM, is consistent with those of the V23, R51 and M maps. The clustering of loci in the W137 map is more extreme than that seen in the other maps (compare Fig. 1), particularly in chromosome VII, where no recombination was observed among 36 of 55 loci, and 43 loci lie within 3.4 cM of one another. We refer to such tight clusters, in which no evidence for recombination was seen, as blocks.

Clusters are not artifacts arising from tandemly repeated DNA elements

With this confirmation of low recombination rates in large blocks of AFLP markers of *P. hybrida*, we sought explanations for their occurrence. Within the tight cluster of chromosome VII, and within others as well, are fragments produced with the same primer set, and thus sharing homology, at least at their termini. For example, E49M36-1a, E49M36-3 and E49M36-4 – all in the chromosome VII cluster exhibiting no recombination – represent short regions of DNA bounded by GAATTCCAG (*Eco*RI site plus CAG) at one end and TTAAACC (*Mse*I site plus ACC) at the other. Although AFLP fragments representing repetitive sequences normally show a very high signal intensity, these fragments were of normal intensity. The formal possibility remained, however, that they represented regions of repetitive DNA containing minor sequence variations. In that case considering each marker as a single locus would artificially exaggerate the extent of clustering. To test the hypothesis that clustered markers produced with one primer pair represent repeated elements, sets of such fragments were excised from AFLP gels and cross-hybridized in Southern blots. Fragments representing the same repeat element were expected to exhibit strong cross-hybridization.

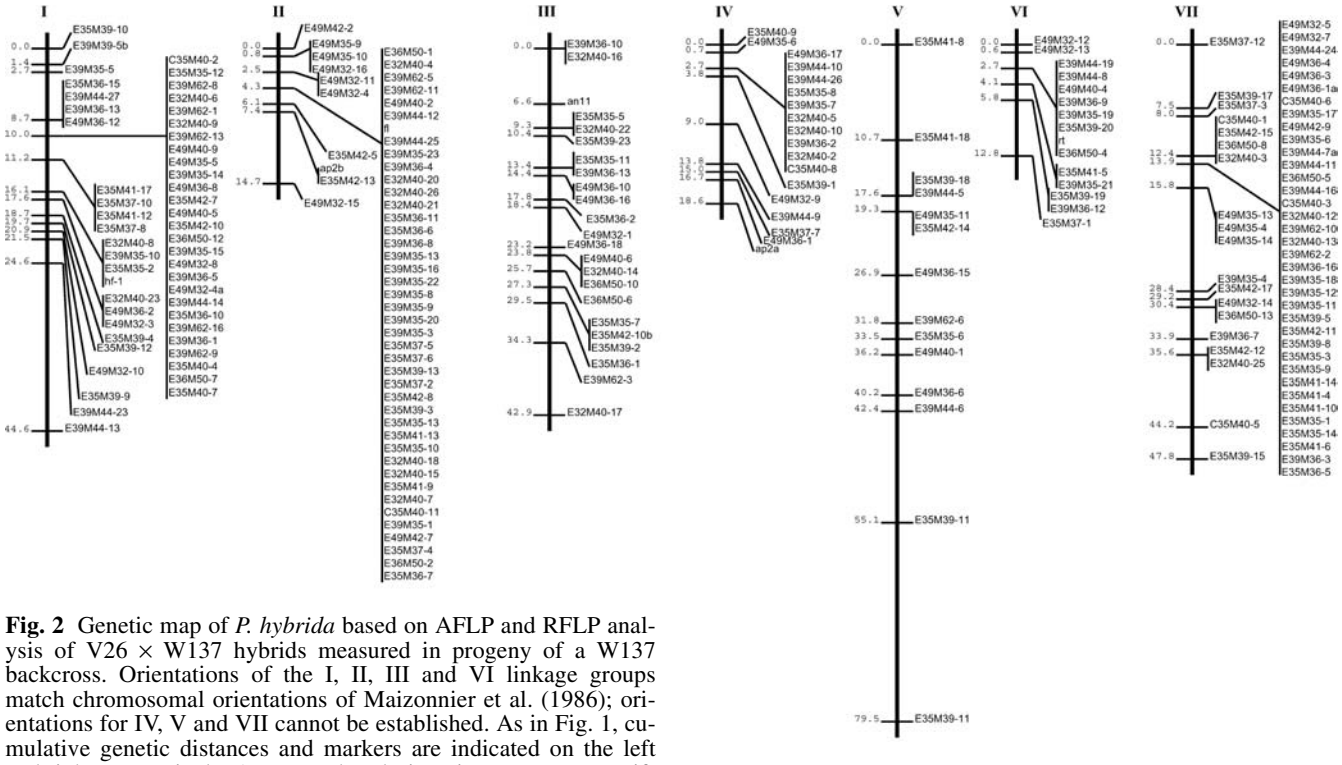

Fig. 2 Genetic map of *P. hybrida* based on AFLP and RFLP analysis of V26 × W137 hybrids measured in progeny of a W137 backcross. Orientations of the I, II, III and VI linkage groups match chromosomal orientations of Maizonnier et al. (1986); orientations for IV, V and VII cannot be established. As in Fig. 1, cumulative genetic distances and markers are indicated on the left and right, respectively. AFLP marker designations are map-specific, and cannot be correlated directly with those of Fig. 1

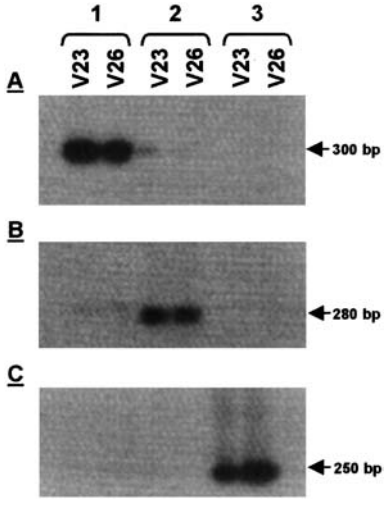

Fig. 3 Southern blot containing co-segregating AFLP products obtained with the primer set GAATTCCAG (*Eco*R1 plus CAG) - TTAAACC (*Mse*1 plus ACC). Pairs of lanes contain products from V23 and V26 mapping gels which appeared to co-migrate, e.g. fragment E49M36(V26)-1a from a V26 mapping gel appeared to comigrate with fragment E49M36(V23)-5 from a V23 mapping gel. The blot was hybridized, in turn, with the following radioactively labeled fragments: *A*: E49M36(V26)-1a; *B*: E49M36(V26)-3; and *C*: E49M36(V26)-4. For an explanation of AFLP marker designations, see Table 2

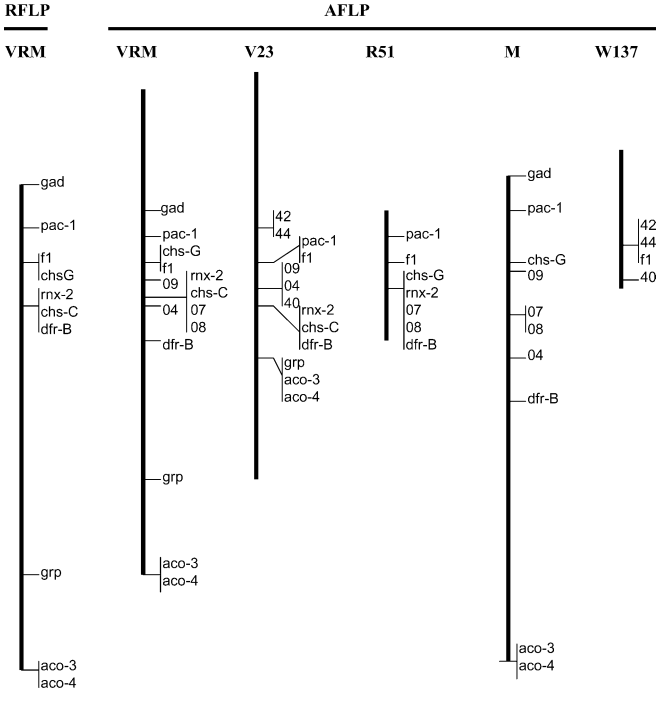

Fig. 4 The original RFLP map of a portion of Ch II (Strommer et al. 2000), together with the equivalent regions of the five AFLP-based maps, showing markers shared by independent maps. In the figure, simple numbers are used to designate shared AFLP fragments, produced by the same primer combination and migrating in similar patterns in multiple AFLP analyses. Full lengths of maps are shown; the placement of markers on each map reflects measured distances in centi-Morgans

Table 4 Frequency of crossovers among 61 individuals comprising the W137 mapping population, by individual chromosomes

Item	Chromosome							Total
	I	II	III	IV	V	VI	VII	
Crossovers per chromosome	Observed number							
0	38	48	36	48	21	53	26	270
1	16	8	19	7	19	6	18	93
2	1	4	4	6	8	1	12	36
>2	6	1	2	0	13	1	5	28
% Of chromosomes with no crossovers	62%	79%	59%	79%	34%	87%	43%	63%
Total # of markers	51	52	23	18	14	15	55	±20 (SD)

Table 5a Markers in high-density clusters as defined by Tanksley et al. (1992). AFLP maps of petunia by chromosome: % in clusters (number of individuals)

Map	Ch 1	Ch ll	Ch III	Ch IV	Ch V	Ch VI	Ch VII	Overall
VRM	0(11)	56(36)	32(22)	0(22)	55(31)	53(15)	54(26)	40(163)
W137	82(52)	90(52)	0(23)	67(18)	0(14)	67(15)	78(55)	68(229)

Table 5b Markers in high-density clusters as defined by Tanksley et al. (1992). AFLP maps of several species

Genome	Predominant marker type	Number of markers	Percent in dense clusters	Reference
Zea mays	AFLP	1,539	7	Vuylsteke et al. 1999
Zea mays	AFLP	1,355	8	Vuylsteke et al. 1999
Arabidopsis	AFLP	321	22	Vuylsteke et al. 1999
Arabidopsis	AFLP	517	23	Alonso-Blanco et al. 1998
Tomato	RFLP	1,030	25	Tanksley et al. 1992
Petunia VRM	AFLP	163	40	This paper
Rye grass	AFLP	471	46	Bert et al. 1999
Sugar beet	RAPD/RFLP	308	51	Nilsson et al. 1997
Petunia W137	AFLP	228	68	This paper

Four tightly linked AFLP fragments bounded by *Eco*RI-CAG and *Mse*I-AAC did not cross-hybridize, and are not, therefore, members of a single repeat family. An additional 16 fragments were probed with a total of eight products obtained with the matching primer pair; and in no cases did the fragments cross-hybridize (data not shown). We conclude that the observed clusters are not artifacts arising from repetitive centromeric elements.

Co-migrating fragments from different varieties represent homologous sequences

We also tested the hypothesis that fragments amplified from different populations but exhibiting similar patterns of migration represent homologous DNA sequences. Three pairs of co-migrating fragments identified between the VR-derived and W137 populations were isolated, re-amplified and analyzed on Southern blots. The expectation was that fragments representing identical loci would cross-hybridize efficiently. The result of one of these experiments is presented in Fig. 3. DNA samples of three E49M36 fragments from a (V26 × W137) × W137 AFLP gel, and the three visually matched fragments from a parallel (V23 × R51) × R51 AFLP gel, were blotted and probed in turn with each of the three V26 fragments.

As can be seen from Fig. 3, the cross-hybridization of co-migrating fragments from different AFLP populations verifies that they represent corresponding loci. This demonstration of a correspondence between the co-migration of fragments from different mapping populations and locus co-identity, matches that reported by van der Voort et al. (1998) in potato. Based on this evidence we identified a number of loci shared by the V23, R51, M and W137 populations and examined the extent of co-localization for these loci. A comparison of co-migrating markers mapped to chromosome II, shown in Fig. 4, typifies what was found for the genome as a whole: while genetic distances between loci may vary among populations, the maps agree in terms of locus order.

Discussion

Our results demonstrate that recombination frequencies and thus map distances in petunia depend greatly on the lines used for mapping. In so far as we can see from the AFLP maps, however, locus order has been conserved among these populations. This is not always the case for petunia, for which there is ample evidence of genome plasticity. Chromosomal assignments for a number of loci have been shown to depend on the lines used, indicating the regular occurrence of translocations (Cornu and Maizonnier 1983; Koes et al. 1987; Fransz et al. 1996; ten Hoopen et al. 1998; Strommer et al. 2000). In reality, therefore, neither genome length nor gene position as determined from one set of crosses can be confidently applied to different lines.

Not only do map distances obtained from V26 × W137 hybrids differ; even maps from V23 × R51 hybrids, produced at different times and with different pollen donors, are variable (Table 3). The variability among V23 × R51-based maps cannot be ascribed to differences in genetic background, which is shared among the three populations. An artificial expansion of genetic maps with increased numbers of markers has been reported, and ascribed to the accumulation of errors in data entry (Becker et al. 1995; Cho et al. 1998; Castiglioni et al. 1999). In our case, however, there is no correlation between marker number and map length.

Clustering of AFLP markers near centromeres has a number of potential explanations. Repetitive elements characteristic of centromeres may produce a number of AFLP markers representing variants of a single repeat, which should be treated as a single marker (Alonso-Blanco et al. 1998; Castiglioni et al. 1998, 1999; Vuylsteke et al. 1999). The demonstrated absence of cross-hybridization among such fragments argues against this explanation. Suppression of recombination is typical of centromeric regions (Beadle 1932; Hartl and Jones 1999; Peters et al. 2001), such that markers are relatively more closely linked in a genetic than in a physical map. This is unlikely to be a complete explanation, however, as we would need to ascribe almost all markers from the V26 × W137 cross to centromeric regions. A third potential explanation, offered by Peters et al. (2001), is that polymorphisms are more often encountered in centromeric regions because they are rich in noncoding DNA, the sequence of which is less constrained. In that work, which assigns AFLP markers to a physical map, a two-fold increase in marker density was found around arabidopsis centromeres. For the same reason as that just cited, however, this cannot provide more than a partial explanation of the extreme clustering characteristic of our petunia AFLP maps.

By definition, marker clustering reflects low levels of crossing-over between the markers. Proper chromosomal segregation in Anaphase-I is generally considered dependent on crossing-over between non-sister chromatids in Prophase-I (Roeder 1997). For each product of meiosis this translates to a minimum of one crossover for every chromosome pair, or at least a 50% probability of finding evidence for recombination on any progeny chromosome. Table 4 summarizes the frequency of crossovers observed for chromosomes in the W137 population, obtained by examining individual genotypes. Overall, for the 427 chromosomes analyzed, covering 228 scorable loci in 61 individuals, only 37% of the chromosomes exhibited evidence of recombination.

There is considerable variation among chromosomes: for chromosomes III, V and VII, we see crossover frequencies of 41–64%, close to or above the theoretical lower limit of 50%. In three other cases, including chromosome II with 52 markers, fewer than a quarter of the progeny chromosomes exhibited evidence for a crossover. Deviations from expected levels of crossing-over have been reported for other molecular maps (Nilsson et al. 1993; Sybenga 1996), but they have involved unexpected abundance, not the paucity seen here. Unless a significant proportion of crossover events are occurring beyond the regions spanned by our markers, *P. hybrida* must feature an exceptional pattern of chromosomal synapsis and segregation.

To quantify the degree of clustering we computed high-density clustering scores, as described by Tanksley et al. (1992). The results are shown in Table 5a. This value, originally applied to a molecular map of tomato comprising 1,030 markers, is obtained by summing the number of markers occurring in clusters of >5 markers per 2 cM and dividing that sum by the total number of markers. The data verify that, for both the VRM and W137 maps, clustering is chromosome-specific, and the unclustered chromosomes differ between lines: chromosomes I and IV of the VRM map, and chromosomes III and V of the W137 map.

Are clustered markers in physical proximity to one another, or are they laid out along an extended region of the chromosome in which crossing-over rarely occurs? Robbins and coworkers (Robbins et al. 1995; ten Hoopen et al. 1996) addressed this question in studies of a line carrying multiple copies of a single transgene. When divergent parental lines were used for mapping, 19 of 47 transgenes on Ch I or Ch II mapped within 1 centi-Morgan of an endogenous phenotypic marker. When mapping was repeated in an inbred background, recombination frequencies between transgenes increased three-fold for Ch I and 12-fold for Ch II. The authors concluded that recombination is suppressed in wide hybrids of petunia (Robbins et al. 1995). The discrepancy between physical and genetic distances was confirmed in subsequent work: six T-DNA insertions lying within 1 centi-Morgan of one another on the genetic map of Ch II were shown by in situ hybridization to span nearly the full length of the chromosome (ten Hoopen et al. 1996). Thus tight genetic linkage represented not physical proximity, but lack of recombination over a significant portion of chromosome II.

Line-specific differences in recombination rates have also been revealed by traditional mapping experiments. For example, *An2* is located at the tip of the short arm of

chromosome VI, and *An1*/*Rt* are located terminally on the long arm. In some combinations of lines they exhibit <1% recombination. Analysis of a set of deletion-derivatives for these loci indicated changes in recombination frequency which correlated with the size of the deletion (Gerats et al. 1984).

Four cytological reports from the 1920s and 1930s provide disparate, and perhaps enlightening, descriptions of pairing in the meiotic chromosomes of petunia. Steere (1932) observed standard terminal pairing of *P. axillaris* chromosomes at diakinesis, in contrast to Matsuda's (1928) description of very loose pairing in *P. violaceae*, the other putative parent of *P. hybrida*. Matsuda frequently observed, in fact, a single ring of 14 chromosomes at diakinesis. Levan (1938), working with inbred *P. hybrida*, reported a prevalence of single chiasma with "early terminalization." Dermen (1931), whose report does not include species or varietal description, saw extremely loose pairing, "so much so that some pairs showed almost no connection between members. Most of the pairs were found loosely attached at one end." There is thus a history of evidence that meiotic pairing in petunia is highly variable, and may differ significantly between the species that generated *P. hybrida*.

We conclude that large clusters found in our maps are likely to reflect loci distributed over a considerable physical distance in which genetic recombination rarely occurs. The low levels of recombination responsible for clustering may be the consequence of the large (phylo-)genetic distance between homologues, as proposed by Robbins et al.; alternatively, they may reflect loose pairing of bivalents characteristic of petunia chromosomes in general or those derived from *P. violaceae* in particular. A propensity for terminal chiasmata would explain our failure to detect the equivalent of even one crossover per bivalent, under the assumption that in these cases the recombination point lay beyond the most-distal AFLP marker.

Maizonnier and his colleagues have identified genes in petunia whose alleles directly affect recombination frequency. The variability between the V23 × R51 and V26 × W137 maps may reflect the actions of alternate alleles for a gene such as rm_1, *recombination modulator* (Farcy et al. 1986; Cornu et al. 1989). Analysis of synaptonemal complexes in Rm_1 and rm_1 lines has shown that the decreased recombination seen in rm_1 homozygotes is accompanied with both incomplete synapsis and reduced chiasma formation (Abirached-Darmency et al. 1992). There is evidence that the genetic effects of rm_1 vary depending on the marker pair studied, a phenomenon which has been ascribed to the action of additional genes acting as modifiers of Rm_1 (Robert et al. 1991). It would be interesting to see if the well-defined rm_1 allele is derived from the *P. violaceae*-like ancestor of *P. hybrida*.

As suggested by Robbins et al. (1995), petunia may not be unique. Most of our understanding of recombination is based on the analysis of a relatively small number of plant species. Meiotic pairing may be weak and recombination levels low in other species, particularly those subjected to human selection. With this issue in mind we computed high-density clustering scores for other AFLP maps in which clustering has been reported. Table 5b presents these data, together with the scores for the VRM and W137 maps of petunia. The level of high-density clustering of markers in the maps of rye grass and sugar beet provides some incentive to take this possibility seriously. In particular one might consider the extent to which suppressed recombination explains cases of linkage drag – the commonly encountered inability to separate unwanted traits from that which a breeder seeks to transfer.

Our results could be seen as evidence that map-based cloning is at present an unrealistic approach for identifying genes of petunia, and perhaps other species as well. Yet from Fig. 4 it can be seen that loci which cluster tightly in one map are likely to be separable in a second. Thus the preparation of multiple AFLP maps, based on different sets of parents, should produce a set of maps which together provide the information required for fine mapping, and eventual map-based cloning. Once AFLP technology is developed, the effort required for additional data generation and analysis is small, and likely to be less daunting and more efficient than alternative approaches.

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