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Origin, distribution and mapping of RAPD markers from wild *Petunia* species in *Petunia hybrida* Hort lines

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Abstract We have established the first linkage map for *Petunia hybrida* based upon both RAPD and phenotypical markers. The progeny studied consisted of 100 BC1 individuals derived from the [(St40×Tlv1)×Tlv1] back-cross. Each morphological marker has previously been mapped onto one of the seven chromosomes. The map consists of 35 RAPD loci of which 24 were affected onto chromosomes while 10 loci were not affected. The loci covered 262.9 cM with a mean distance of 8.2 cM. They are dispersed over seven linkage groups, of which six are carried on identified chromosomes. The RAPD markers were also applied on a set of ten *P. hybrida*, lines chosen for their diversity and on a set of seven wild species corresponding to the possible ancestors of the *P. hybrida* species. The markers were found both in the wild species as well as in *P. hybrida* lines indicating that they are inherited and are stable enough to establish similarities and to suggest relationships between species. Eight out of the ten lines carry different linkage groups of RAPD markers, which suggest that recombinant events occurred between chromosomes which originated in the wild species.

Key words Petunia · RAPD · Genetic map · Origin Chromosome blocks

Introduction

In the seventies, *Petunia hybrida* was considered to be a model plant for genetic analysis using traits such as pigment colours and chlorophyll deficiencies (Bianchi and Dommergues 1979; Wiering et al. 1979; Hanson 1980; Cornu 1984) and extensive studies were also performed on both the chloroplastic (Palmer et al. 1983; Sytsma and Gottlieb 1986) and mitochondrial genomes (Folkerts and Hanson 1989). RFLP mapping in *Petunia* has previously been carried out for some identified genes, namely the actin gene families and the chalcone synthase genes (Koes et al. 1987; McLean et al. 1990). A combination of cytogenetics and genetic linkage mapping has allowed the construction of a genetic and cytological map. For *P. hybrida*, five out of seven chromosomes are cytologically identifiable (Maizonnier 1976, 1984). A complete primary trisomic set is available and which has facilitated the assignment of several traits (Maizonnier and Moessner 1979) while breakage-fusion-bridge cycles (leading to translocation or ring chromosomes), functioning at mitosis and meiosis, have contributed to establishment and further completion of a cytogenetic map (Maizonnier and Cornu 1971, 1979). Recent progress has been made with the deletion mapping technique and the use of telotrisomics (Maizonnier et al. 1986) and to-date the cytological map contains 26 markers. Up to now about 120 traits have been recorded and assigned to chromosomes using both cytogenetics and genetic linkage analysis (Cornu et al. 1990), but three-point-mapping with such traits is time consuming and expensive and actually only 20 of them can be ordered along chromosomes (Cornu et al. 1980).

Recently, two complementary lines were constructed which carry different alleles at one locus on each chromosome pair, all affecting flower pigmentation. Thus, genetic linkage with one of these traits and a new marker, either phenotypic or molecular, can be easily detected enabling us to assign it to the corresponding chromosome. In order to establish an ordered genetic map for *P. hybrida* we chose an assay based on the amplification of random DNA segments with 10-mer primers, now available from Operon Technologies Inc. (Williams et al. 1990). Several assays on different plant species have been performed to detect polymorphisms between the amplification products of different individuals (Klein-Lankhorst et al. 1991; Vierling and Nguyen 1992). Moreover, these polymorphisms have

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Table 1 List, code, traits and origins of the plant materials

Species	Code	2n=	Colour	Origin
P. parviflora		18	Purple	USA Michigan[a]
P. linearis	S11	18	Violet pink	IGA[d] Origin Corrientes Argentine 1980.
P. parodii		14	White	USA Michigan[a]
P. integrifolia	S13	14	Violet pink	IGA[d]
P. axillaris	M1	14	White	USA Cornell[b]
P. inflata	DII	14	Purple	Dijon
P. violacea	S10	14	Purple	IGA[d]
P. hybrida	lines	14		
	Ta2		Purple	Cultivar "Velours rouge"
	Tlh1		Diluted violet	Doubled Haploïd Tl67
	Sk176b		Pale pink	Mutagenesis on cultivar "Satin rouge"
	Pc6		Magenta	Cultivars "Salmon Gem", "Moonstone", "Velours Rouge"
	Tlv1		Diluted violet	Cultivars "Salmon Gem", "Velours Rouge"
	Tb1-3		Pourpre	Cultivar " Velours rouge"
	St40		Very pale pink	IGA R40 Cultivars "Fire Chief", "Rose du ciel", "Violet"
	St43		Violet pink	IGA R43 Cultivars "Fire Chief", "Rose du ciel", "Violet"
	Tlh7		Violet white	Double haploid: Mitchell "Rose du ciel"×*P.axillaris*
	Rlkh1		White	(Tlv1×St43)×St43 doubled haploid[c]

[a] K. C. Sink, East Lansing (USA)
[b] M. Hanson, Cornell University (USA)
[c] C. Raquin, Orsay (France)
[d] IGA: Institute of Genetics, Amsterdam (The Netherlands)

been shown to be useful for analyzing genetic linkage relationships in a wide range of plant species (Sederoff and Neale 1991; Echt et al. 1992 ; Reiter et al. 1992). Because doubts remain as to the stability and repeatability of RAPD markers, used either in genetic diversity studies or in genetic mapping, we studied the origin of RAPD markers in specimens of the wild species of *Petunia* from which *P. hybrida* is believed to have originated about 170 years ago (Wijsman 1982).

We report here the methods for screening primers for amplification of DNA segments available for mapping. We produce the first multipoint genetic map covering 262.9 cM with an average spacing of 8.2 cM. This genetic map is constructed for the line St40, but the primers were also verified for their use in a set of ten *P. hybrida* lines covering the large range of genetic diversity known for this species. We revealed blocks of markers in lines and concluded that these blocks were partially maintained after several crosses.

Table 2 Genotypes of Tlv1 and St40 for marker traits

Lines	Chromosome						
	I	II	III	IV	V	VI	VII
Tlv1	Hf1	fl	Ht1	ph2	po	an2	an4
St40	hf1	Fl	ht1	Ph2	Po	An2	An4

Hf1, hydroxylation in 3′ and 5′ of anthocyanins
hf1, hydroxylation in 3′ of anthocyanins
Fl, enhanced synthesis of flavonols in flowers
fl, reduced synthesis of flavonols in flowers
Ht1, Quercetin as main flavonol in flowers
ht1, Kaempferol as main flavonol in flowers
Ph2, pH of corolla homogenate about 5.5
ph2, pH of corolla homogenate about 6
Po, without yellow pigment in pollen
po, with yellow pigment in pollen
An2, coloured corolla
an2, very-light-coloured corolla
An4, anthocyanin in pollen
an4, without anthocyanin in pollen

Materials and methods

Plant material

The plant material is described in Table 1. Plants were grown in a glasshouse at 20 °C for a 16 h day and 15 °C at night. Lines carrying alternative alleles on each chromosome, St40 and Tlv1, were backcrossed in controlled conditions (the genotypes of these lines and the function of the genes involved are presented in Table 2). One hundred individuals of the BC1 population [(St40×Tlv1)×Tlv1] were used for genetic linkage analysis and were maintained in vitro. Lines were chosen from the Dijon collection in order to maximise the genetic diversity. The first criterion was to obtain lines originating from different cultivars and the second criterion was to choose material in collections constructed by different breeders (Amsterdam and Dion) in order to diversify the original sources of germplasm. Species and lines of *P. hybrida* were maintained inbred and one individual per species or line was sampled for analysis.

DNA preparation

DNA was prepared from young leaves of two to three month-old plants according to Bernatsky and Tanksley (1986) except that the Cesium chloride purification step was omitted. Final concentrations of total DNA were adjusted to 20 ng/µl. The same extracts were used throughout the experiments.

Amplification protocol

The amplification procedure took place in 25 µl of 67 mM Tris-HCl pH 8.8, 3 mM $MgCl_2$, 17 mM ammonium sulphate, 500 M of each dNTP, 30 ng of primer and 1.5 units of *Taq* polymerase (Boehringer). Each reaction was overlaid with 50 µl of paraffin oil to prevent evaporation. Samples for amplification were subjected to 30 repeats

of the following thermal cycles: 1 min at 92 °C, 1 min at 38 °C and 1.5 min at 74 °C on a Biometra trioblock thermocycler with the fastest transition time between the different steps. Fragments generated by amplification were separated according to size on 2.2% agarose gels run in 1×TAE (40 mM Tris acetate pH 7.8, 1 mM EDTA), for 4 h at 5 V/cm, stained with ethidium bromide and visualized by transillumination with ultraviolet light (312 nm).

Probes

Mapped RAPD fragments from the St40-line DNA were used as probes onto the Southern transfer of the whole amplification products obtained with the DNA of the different species and lines of *P. hybrida*. These fragments were recovered from agarose gels according to the technique described by Tautz and Rentz (1983). Recovered DNA was labelled by random priming with ^{32}P-dCTP (3000 mCi/mmole) using the random primed DNA labelling kit from Boehringer. Hybridizations were performed in 6 SSC ; 5× Denharts; 0.1% SDS overnight at 65°C. Filters were rinsed for 20 min at 65°C in 2 SSC plus 0.1% SDS and then 20 min at 65°C in 0.2 SSC plus 0.1% SDS. Autoradiograms were obtained after 15 min to 2 h exposure at –70°C.

Construction of a linkage map

The map was established using the UNIX version of Mapmaker 2.0 on a SUN Sparcstation (Lander et al. 1987; Lincoln et al. 1990). The entire set of markers was processed using two-points (LOD >3), three-point and multipoint analysis. A core map was established using the markers which were ordered with a Lod score >3.

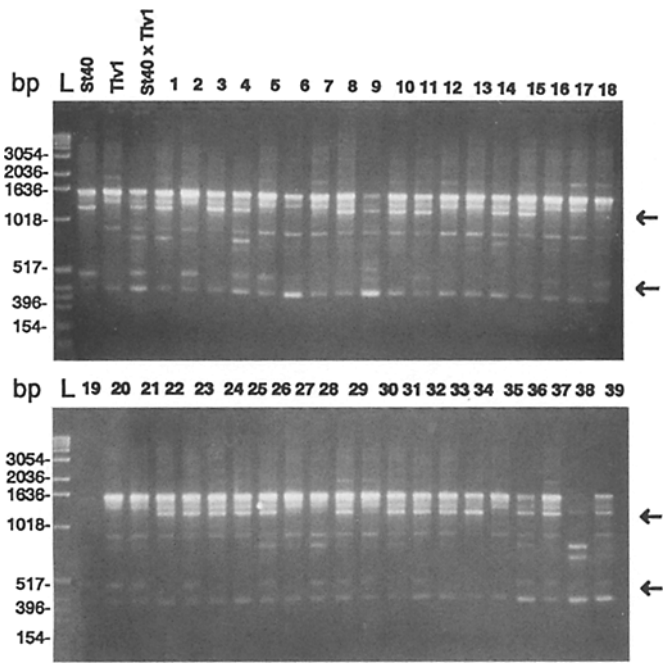

Fig. 1 Segregation among 39 individuals of the $OPJ6_{1500}$ and the $OPJ6_{500}$ fragments (*arrows*). Lane L, the 1-kb ladder from Bethesda Research Laboratories served as a reference for the size of fragments. *Lanes 1 to 39* correspond to segregant individuals

Results

Primer selection

The primers were screened against St40, Tlv1, and the hybrid, for the presence of at least one fragment in St40 and in the hybrid, with no corresponding fragment in Tlv1. After screening, 18 out of the 80 primers assayed were retained.

Linkage mapping

Petunia DNA was prepared from segregant individuals and controls (St40, Tlv1, F1). The complete analysis using the 18 primers determined the segregation patterns for 35 amplification products. On a gel obtained as in Fig. 1, the segregation of fragments $OPJ6_{1500}$ and $OPJ6_{500}$ among 39 individuals is readable. Genotypic determination for seven marker traits was performed in the glasshouse after flowering for the Fl, Po, An2, and An4 markers or after tests of BC1 plants for the hf1, ht1 and ph2 markers. The complete results, and the corresponding chi^2 for an expected segregation ratio of 1:1, are summarized in Table 3. One marker shows significant deviation at the 1% level to the expected 1:1 ratio and ten markers deviate at the 5% level. Because the two lines carry one marker trait already assigned per chromosome (Cornu et al. 1990), the linkage groups were expected to be assigned to the corresponding identified chromosomes. Six groups have been assigned to six different chromosomes. One important group (57.7 cM) had no significant linkage with any trait marker and so remained unassigned. Two RAPDs remained independent from any linkage group (Fig. 2).

The linkage map obtained with 42 markers in a multipoint analysis for 100 BC1 individuals is the most complete one for *P. hybrida* to-date. The order of 21 markers is defined with a LOD score >3. The total length of continuing markers is 262.9 cM with an average distance between successive markers of 8.2 cM. Assuming an average length of 100 cM per chromosome the proportion of the genome covered rate would at most be 37%.

Origin of the mapped RAPD makers

Seventeen primers were further tested with a set of seven wild *Petunia* species and on ten *Petunia hybrida* lines (Table 1). Figures 3 A and 4 A show the amplification products obtained with the primers OPJ6 and OPV8 respectively. The mapped polymorphic fragments are $OPJ6_{1500}$ and $OPJ6_{500}$, $OPV8_{1500}$, and $OPV8_{600}$. Hybridizations were performed in order to establish that fragments of the same size in two individuals shared homology. We always performed hybridizations with the mapped fragment, recovered after St40 DNA amplification, as a probe. After hybridization with the $OPV8_{1500}$ fragment we established that $OPV8_{1500}$ is present in *P. parodii*, *P. integrifolia*, *P. inflata*, *P. violacea* and in *P. hybrida* lines Tb1-3, Tlh7, and St40 (Fig. 4 B). For fragment $OPJ6_{500}$ a strong band is present in most of the lanes while a weak band of the same size is present in Tlh1, Tlv1 and Rlkh1, although the marker is absent in *P. linearis*, *P. parviflora*, *P. axillaris* and Tlh7

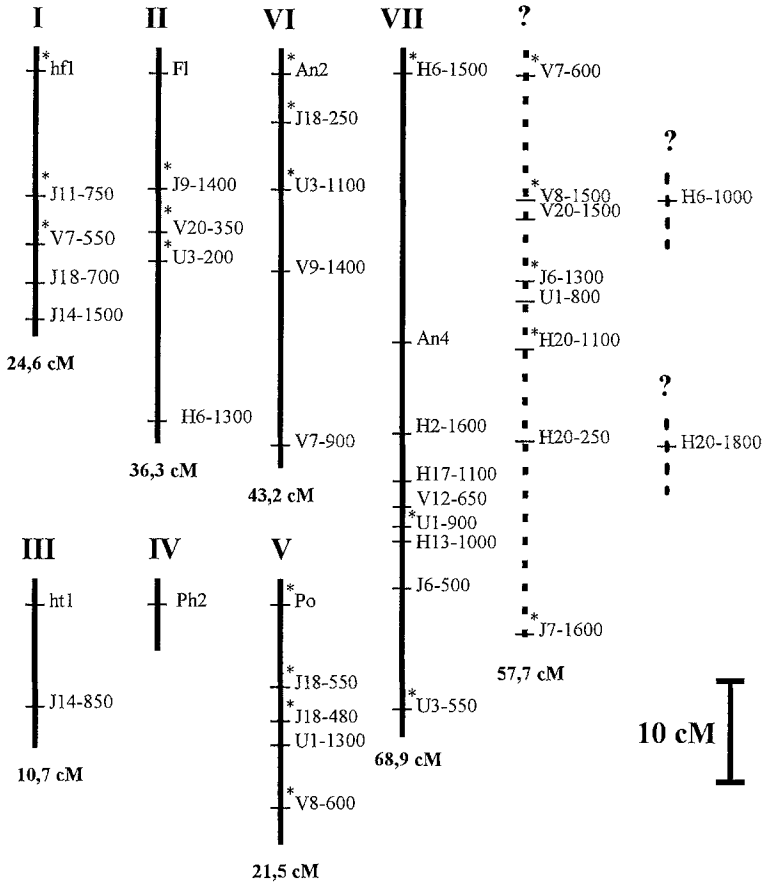

Fig. 2 Linkage map of the St40 line including seven phenotypical markers, six identified chromosomes carrying a linkage group, one chromosome with no molecular marker, and one unaffected linkage group. An * indicates markers ordered with a LOD score >3

Table 3 Chi-square values according to the expected 1:1 segregation ratio for each marker mapped
A: Markers carried by identified chromosomes

I		II		III		IV		V		VI		VII	
hf1	1,71	Fl	2,47	ht1	0,1	pH2	0,51	Po	0,39	An2	5,26*	An4	2,13
J14-1500	4,05*	H6-1300	4,84*	J14-850	2			V8-600	0,375	V9-1400	1,25	J6-500	0,25
J11-750	6,12*	J9-1400	6,57*					U1-1300	0,375	V7-900	0,66	H17-1100	0,53
V7-550	6,44*	V20-350	6,31*					J18-480	0,25	U3-1100	2,13	H13-1000	0,85
J18-700	4,45*	U3-200	9,37**					J18-550	0,01	J18-250	4,45*	H6-1500	0,09
												H2-1600	0,68
												V12-650	1,74
												U1-900	1,27
												U3-5500	0,09

B: Markers carried by unidentified chromosomes

unmapped linkage group		not linked marker		not linked marker	
J6-1300	1,04	H6c	0,09	H20a	0,26
J7-1600	0,01				
H20-1100	0,16				
H20-250	4,45*				
V8-1500	0,52				
V20-1500	0,51				
V7-600	0,16				
U1800	0,16				

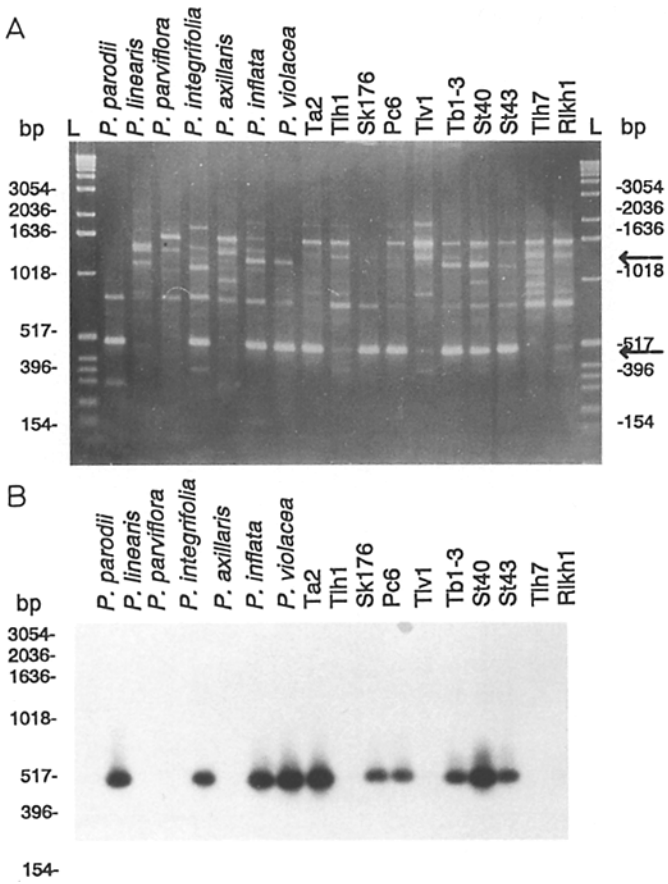

Fig. 3 A A 2.2% agarose gel, stained with ethidium bromide (0.1 µg/ml), of PCR amplification products with the OPJ6 primer. *Arrows* indicate mapped RAPDs. The 1-kb ladder from Bethesda Research Laboratories served as a reference for the size of fragments. **B** Southern transfer hybridized with the $OPJ6_{500}$ PCR-amplified fragment from the St40 line

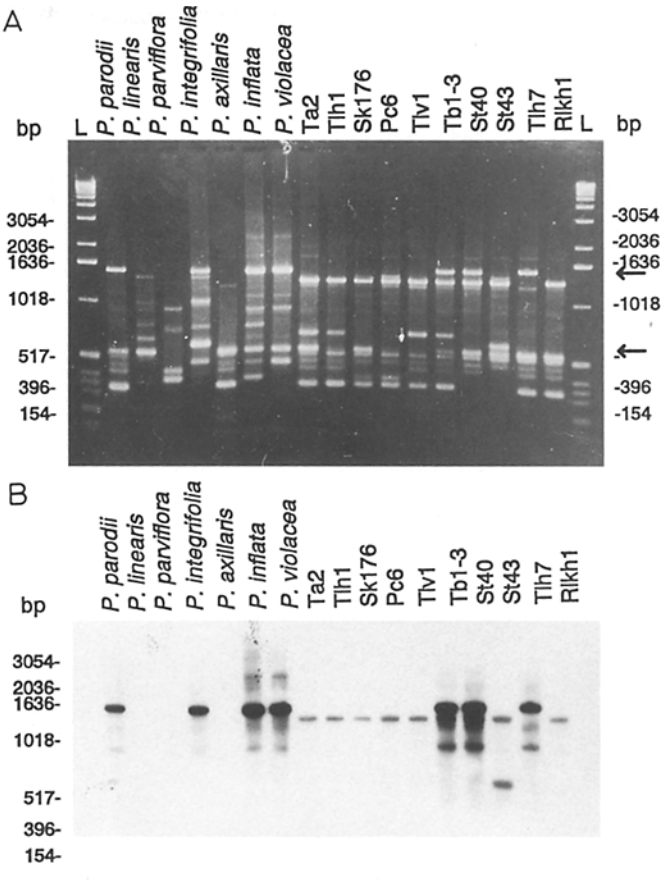

Fig. 4 A A 2.2% agarose gel, stained with ethidium bromide (0.1 µg/ml), of PCR amplification products with the OPV8 primer. *Arrows* indicate mapped RAPDs. The 1-kb ladder from Bethesda Research Laboratories served as a reference for the size of fragments. **B** Southern transfer hybridized with the $OPV8_{1500}$ PCR-amplified fragment from the St40 line

(Fig. 3 A). The absence of a signal in the Tlv1, Tlh1, Rlkh1 lanes shows that the strong and weak bands do not share any homology in sequence (Fig. 3 B) and, moreover, that the weak band is absent in the probe and hence in the St40 DNA amplification products. During our work we performed some repetition of amplifications, either in the segregation studies or, in the application of the RAPD markers to the putative ancestral lines of *P. hybrida*. We noted that the reproducibility in terms of the stoichiometry of fragments was poor from one amplification to another, although the general pattern of bands was reproducible. A comparison of the amplification product obtained with primer OPJ6 and St40 DNA (Figs. 2 and 3 A) reveals the different intensity of fragment $OPJ6_{500}$. The same sample of DNA was used in the two cases. The intensity of fragment $OPJ6_{500}$, when present, is reproducible from one individual to another throughout the two experiments. These kinds of variations could result either from small differences in the composition of the mix, realized prior to the reaction, or else to small variations in the temperature profile along the cycles from one experiment to another one (Penner et al. 1993). In order to resolve the discrepancies concerning the presence and homology of some of the mapped fragments, we performed hybridizations with 20 out the 35 mapped fragments (Fig. 5).

In this way we built up a map of St40 according to the origin of fragments (Fig. 5). Thirty out of thirty-five mapped RAPDs can be found in at least one of the different species. For instance chromosome VII carries markers present in all species except *P. linearis* and *P. parviflora,* while chromosome II carries markers from *P. axillaris*, one of which is also present in *P. parodii*. Moreover, *P. linearis* and *P. parviflora* did not contribute to the St40 mapped marker set. The origins of chromosome markers can be exclusive between each species (Fig. 5), common to one group (white versus coloured flower), or else conserved between the different species.

We examined the presence of the St40 mapped RAPDs in the nine other lines according to both size and homology. The results are summarized for each line, per locus per chromosome, according to the molecular map built up for St40 (Table 4). Some markers are isolated depending on the line in which they are found. However, we observed that the St40-linked structures may be generally conserved,

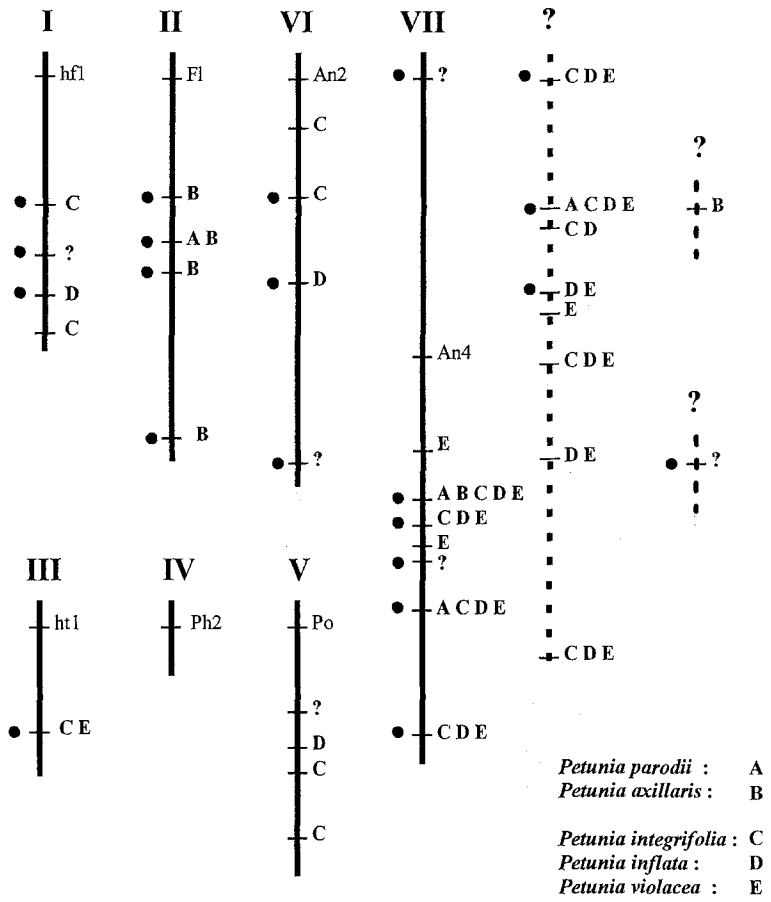

Fig. 5 Origin of the mapped markers; ● according to hybridization results with the PCR fragments as a probe; no specification, means according to the size only. ?, indicate markers not found in our species set

although shortened among lines, and may even be totally absent.

Discussion

About 120 traits have been assigned to the seven chromosomes of *P. hybrida*. Generally independent two-point tests have been performed, resulting in the marker traits being assigned to the seven linkage groups, although without accurate mapping. The construction of lines in order to build three-points tests with classical traits is space and time consuming. RAPD technology has allowed the rapid construction of an ordered map and at a reasonable cost in terms of materials and money. This map can also be used for other genetic studies.

The inheritance of RAPDs as dominant traits led us to use BC1 progeny for the mapping experiment. We observed some distortion in segregation for markers assigned to chromosomes I and II because of the excess of St40 alleles, in the maternal non-recurrent parent. Such bias has been encountered with RFLP in lettuce (Landry et al. 1987) and lentils (Havey et al. 1989) and has already been observed for traits in *Petunia* (Cornu, personal communication). The most likely explanation of this bias in a BC is selection favouring heterozygous genotypes.

The five 2n=14-chromosome wild species are broadly related in two branches; one includes *P. parodii* and *P. axillaris*, both with white flowers, while the other includes *P. integrifolia*, *P. inflata* and *P. violacea* all of which have coloured flowers (Wijsman 1982). All of these species can be intercrossed with various levels of success and show no strict incompatibility. Botanists consider that *P. hybrida* lines were derived from interspecific crosses, performed 170 years ago, between a coloured and a white flowered species recognized respectively as *P. integrifolia* and *P. axillaris* (Stout 1952; Sink 1984). Our results demonstrate that sequences amplified with short primers can be inherited between species and could, therefore, represent good markers from ancient introgressions.

P. hybrida is a young species so that mutations could not explain the presence of polymorphism between lines in such a short time. Therefore, most of the identified polymorphism must have originated earlier. Only 4 out of 35 mapped RAPDs are found exclusively in the white flower species. The 18 primers used for mapping identify 20 RAPDs specific for Tlv1, versus St40, that could be useful for mapping in the cross [(Tlv1×St40)×St40]. Eighteen of these are present exclusively in the white-flower species. This symetry leads us to consider that polymorphisms involve chromosomal regions which originate from different groups of species. Consequently, we propose that the RAPD polymorphisms found in *P. hybrida* appeared

Table 4 Chromosome blocks found in *P. hybrida* lines according to the origin of markers in Paxi (*P. parodii* and *P. axillaris*) or Pint (*P. integrifolia*, *P. inflata* and *P. violacea*)

CHR	Markers	Lines									
		St40	St43	Ta2	Sk176b	Pc6	Tb1-3	Rlkh1	Tlh7	Tlh1	Tlv1
I	Hf1			+			+		+	+	+
	$OPJ11_{750}$	Pint	+	+							
	$OPV7_{550}$	?	+	+	+						
	$OPJ18_{700}$	Pint	+	+						+	
	$OPJ14_{1500}$	Pint	+	+							
II	Fl	Paxi							+		
	$OPJ9_{1400}$	Paxi									
	$OPV20_{350}$	Paxi							+		
	$OPU3_{200}$	Paxi							+		
	$OPH6_{1300}$	Paxi			+				+		
V	Po	Pint									
	$OPJ18_{550}$	?	+		+			+			
	$OPJ18_{480}$	Pint	+		+			+			
	$OPU1_{1300}$	Pint	+		+			+			
	$OPV8_{600}$	Pint									
VI	An2	Pint	+	+	+	+	+				
	$OPJ18_{250}$	Pint	+	+	+	+					
	$OPU3_{1100}$	Pint	+	+	+	+					
	$OPV9_{1400}$	Pint	+	+	+	+					
	$OPV7_{900}$	?	+	+	+	+			+		
	$OPH6_{1500}$	?									
VII	An4	Pint	+	+							
	$OPH2_{1600}$	Pint	+	+							
	$OPH17_{1100}$	Paxi/Pint	+	+							
	$OPV12_{650}$	Pint	+	+							
	$OPU1_{900}$	Pint		+							
	$OPH13_{1000}$	?		+							
	$OPJ6_{500}$	Paxi/Pint	+	+							
	$OPU3_{550}$	Pint	+	+							
?	$OPV7_{600}$	Pint	+	+							
	$OPV8_{1500}$	Paxi/Pint					+				
	$OPV20_{1500}$	Pint	+				+				
	$OPJ6_{1300}$	Pint					+				
	$OPU1_{800}$	Pint					+				
	$OPH20_{1100}$	Pint					+				
	$OPH20_{250}$	Pint					+				
	$OPJ7_{1600}$	?									

+, undetermined origin

between two genetic pools. They are summarized as Paxi for *P. axillaris* and *P. parodii*, and Pint for *P. integrifolia*, *P. violacea* and *P. inflata*, respectively. We suggest that *P. hybrida* resulted from crosses betweeen these two genetic pools.

St40 contains five chromosomes and one unaffected linkage group carrying mapped RAPDs from at least one out of three coloured species, and one chromosome carrying markers found only in Paxi. Three mapped RAPDs out of thirty-five are found conserved between Pint and Paxi and could have originated before the separation of the two genetic pools. Other RAPDs can be found in one or more species of a genetic pool. The inbred lines used to represent species could not be considered as representative of the whole diversity present in the species. Therefore, when two neighbouring RAPDs are found in a linkage group in two different species we cannot determine whether this arrangement results from genetic exchange between the species or if the markers are present in the two species. In St40 we did not find mixed arrangements of markers belonging to the two genetic pools.

We observed that the primers are stable and had been inherited as a whole in the St40 line at the time of introgression. We can consider that most of the polymorphism found in *P. hybrida* result from ancient introgressions occurring in the early interspecific crosses. Consequently, we examined the organization of alleles which originated from introgressions in *P. hybrida*. We looked at the neighbouring fragments of each fragment to determine whether or not chromosome blocks are generally maintained, and therefore inherited, in lines (Table 4). When a marker can originate in two genetic pools we applied the parsimony principle and considered that the origin of this marker in a line is identical to that of its neighbour. It is clear that blocks

of RAPD markers either from Pint or from Paxi are maintained in *P. hybrida* lines. Nevertheless, in BC1 individuals we found some structure resulting from one, two, or even three cross-over events between chromosomal segments of different origin (Paxi versus Pint) in the mapped region. These kinds of events could have occurred during the breeding process over the 170 last years. Considering the origin of polymorphism between the two genetic pools, the dominant character of RAPDs and the method of screening for RAPDs would lead to chromosomes presenting an alternating presence and absence of RAPDs. The result on a line would depend on the genetic pool in which the chromosomal fragments, and consequently the RAPDs, originate. Nevertheless, mixed arrangement of markers from different origins are rarely found in lines. Consequently, we believe that there was a strong selection pressure against these recombinant structures and, conversely, a strong selection pressure to conserve blocks. These selection pressures are most likely due to abnormal meiotic behavior between recombinant chromosomes from different pools and to fitness for linked loci. With such an origin of polymorphisms and the block organization of introgression, two polymorphic regions in which homologous chromosomes are of different origin, separated by a non-polymorphic one in which homologous chromosomes are of the same origin, will either exhibit a high recombination rate or be apparently independent because of the lack of intermediary markers. Moreover, preferential selection of conserved blocks in a population limits the possibility for the creation of a new allelic arrangement developed from different genetic pools.

We believe that most species have developed polymorphisms from introgression of the kind we have observed in *P. hybrida* lines. Our experimental design recognizes the origin of polymorphisms by introgression and we point out that such introgression introduces chromosomal blocks. The natural occurrence of introgression in crops may have led to equivalent blocks which introduce discrepancies in genetic maps and reduce the expected allelic combination. RAPD markers represent a powerful and convenient tool to determine the origin and structure of such introgressions.

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