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Comparison between *Poncirus* and *Citrus* genetic linkage maps

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Abstract Five genetic linkage maps were constructed for the parents of three progenies: *Citrus aurantium* (A) × *Poncirus trifoliata* var. *Flying Dragon* (Pa), *C. volkameriana* (V) × *P. trifoliata* var. *Rubidoux* (Pv) and a self-pollination of *P. trifoliata* var. *Flying Dragon* (Pp). The number of polymorphic markers assayed ranged from 48 for Pa to 120 for A according to the heterozygosity of each parental. As our focus was on genome comparison, most of the markers were newly generated simple sequence repeats. Inter-retrotransposon amplified polymorphisms based on four retrotransposon sequences isolated from *Citrus* spp were also used to saturate the maps. These polymorphisms were much more frequent in A (53) than in Pa (15) and randomly distributed throughout both genomes. Since comparative genomics and quantitative trait locus analysis applicability depends on the reliability of marker ordering, the causes of variation in marker order were investigated. Around 25% of the markers showed gametal segregation distortions. Segregation distortions were also observed at the zygotic level towards a reduction in the observed frequency of homozygotes from that expected in linkage groups 5 and 7. The presence of balanced lethal factors or gametal incompatibility genes in those genomic regions would explain a zygotic advantage of heterozygotes at these specific regions. Four differences in genomic organization were observed; three are putative translocations and affect homeologous linkage groups 3, 7 and 11, where highly distorted markers are found. Other causes of variation in marker order are also discussed: the introduction of new markers in the map, lowering the LOD score and the mapping software. These results represent the first comparative mapping analysis among *Citrus* and *Poncirus* species.

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Introduction

Citrus trees are economically the most important fruit trees on an international scale, with an annual production exceeding 95 million tons (FAO 1999). Citrus cultivars belong to several species, in fact, the fruit originates from a scion which is grafted onto an apomictic rootstock propagated by seed to ensure uniform production and freedom from diseases. The scion belongs mainly to the following species (or crosses between them): *Citrus sinensis* (L.) Osb. (sweet orange), *C. clementina* Hort. ex Tan. (clementine mandarin), *C. unshiu* (Mak.) Marc. (satsuma mandarin), *C. paradisi* Macf. (grapefruit) and *C. limon* L. Burm. f. (lemon). Other species are used as rootstock, or in rootstock improvement programs, such as *Poncirus trifoliata* (L.) Raf. (trifoliate orange), *C. aurantium* L. (sour orange), *C. volkameriana* Ten. (Volkamer lemon) and *C. reshni* Hort. ex Tan. (Cleopatra mandarin). While a wide diversity of scion cultivars exists, the number of available rootstock cultivars is very limited in several countries. For example, in eastern Spain, more than 85% of new sweet orange and mandarin varieties are grafted onto just one rootstock genotype, the citrange Carrizo (*C. sinensis* × *P. trifoliata*) (Pina et al. 2000), resulting in its vulnerability to attack from new pathogenic agents, or the evolution of existing ones into more virulent forms, or the progressive salinisation of arable lands.

The genetic improvement programs of citrus focus on obtaining new rootstocks that are resistant to disease and better adapted to adverse growing conditions as well as diversifying the limited choice of currently available rootstocks. Such improvement must be based on a knowledge of the genetic control of the potential traits and the use of plant genetic resources. The group of true citrus fruit trees includes six genera, *Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus* and *Citrus*. All

Table 1 Citrus linkage maps already published. *N* Number of individuals that form the analyzed family, *centiMorgans* total size of the linkage map, *M* number of markers, *LG* number of linkage groups forming the map

Reference	Family	N	centiMorgans	M	LG	Software
Jarrell et al. (1992)	Sacaton × Troyer	60	351	38	10	MAPMAKER
Durham et al. (1992)	*C. grandis* × *P. trifoliata*	65	553	52	11	MAPMAKER
	C. reticulata × *C. paradisi*	65	314	32	8	MAPMAKER
Cai et al. (1994)	*C. grandis* × *P. trifoliata*	60	1,192	189	9	MAPMAKER
Luro et al. (1996)	*C. grandis*	52	600	34	7	MAPMAKER
	C. reshni × *P. trifoliata*	52	1,503	95	12	MAPMAKER
Kijas et al. (1997)	Sacaton × Troyer	57	410	48	12	JOINMAP
Simone et al. (1997)	*C. aurantium*	50	1,000	247	20	MAPMAKER
	C. latipes	50	600	92	12	MAPMAKER
Garcia et al. (1999)	*C. volkameriana*	80	137	45	9	JOINMAP
	P. trifoliata	80	126	38	5	JOINMAP
Cristofani et al. (1999)	*C. sunki*	80	867	63	10	MAPMAKER
	P. trifoliata	80	732	62	8	MAPMAKER
Roose et al. (2000)	Sacaton × Troyer	57	701	153	16	JOINMAP

of these genera have persistent unifoliolate leaves except for the monotypic genus *Poncirus*, which has trifoliolate, deciduous leaves. In addition to *Poncirus*, *Clymenia* and *Eremocitrus* are also monotypic genera. *Fortunella* and *Microcitrus* have four and six species, respectively. *Citrus* consists of the largest number of species (Swingle 1943). The construction of genetic linkage maps would permit the organization of their different genomes to be compared as a first step towards an efficient and continuous use of plant genetic resources to enrich the gene diversity of breeding programs.

Mapping in outbreeding heterozygous perennial crops is not as advanced as in annual crops. The former require more time and more space, given the long growing cycle and large crop size. Only progeny from the cross between two, more or less, heterozygous parents is usually available. In this case, up to four alleles per locus may segregate, and the marker phase (coupling or repulsion) can not always be deduced from parent and grandparent banding patterns.

To date, 14 genetic maps have been published (Table 1), but no comparison among linkage groups of *Citrus* and *Poncirus* species has been reported. From the 14 published maps, only a few correspond to recognized species: *C. grandis* (Luro et al. 1996), *C. aurantium*, *C. latipies* (Simone et al. 1998), *C. volkameriana*, *C. sunki* and *P. trifoliata* (Cristofani et al. 1999; García et al. 1999). In other cases, just one consensus map has been obtained for the two parents without taking into account the possible chromosomal reorganizations which have come about in the evolution of the family *Aurantioidae* (Naithani and Raghuvanshi 1958; Raghuvanshi 1962; Herrero et al. 1996; Garcia et al. 1999) or because the family design (a cross between two inter-generic hybrids) did not permit anything else. In some cases, the genetic map has been obtained mainly with dominant markers (random amplified polymorphic DNAs, inter simple sequence repeats, etc), and, therefore, although we are dealing with the same primer, one cannot be certain that a similar-sized band corresponds to the same marker locus in different populations.

For these reasons, an initial objective of the present work was to develop new codominant markers in citrus in order to be able to make comparative analyses of genome organization feasible using adequate number of progeny. Microsatellites are simple sequence repeats (SSR); these are very abundant in eucariots and display high polymorphism (Tautz and Renz 1984; Wang et al. 1994; Russell et al. 1997). They are randomly distributed in the genome, both inside and between genes. The microsatellites have a mutation rate per generation that varies between 2.5×10^{-5} and 2.5×10^{-2} and are therefore the most quickly evolving DNA sequences (Kashi et al. 1990; Weber and Wong 1993).

As well as microsatellites, we have also developed IRAPs (inter-retrotransposon amplified polymorphisms). Due to the abundance of retrotransposon sequences in the genome in many plant species they have been used in studies of phylogeny, biodiversity and linkage (Brandes et al. 1997; Ellis et al. 1998). In the case of citrus, the retrotransposon sequences corresponding to the family Ty1-*copia* are very abundant (Asins et al. 1999) and present a greater level of polymorphism than any other type of sequence (even microsatellites) in the vegetatively propagated crop *C. clementina* (Bretó et al. 2002). Therefore, using these sequences will supply a large number of highly reproducible markers, both simply and quickly, to saturate the citrus maps.

We report here the construction of five genetic maps using three segregating populations derived from three species commonly involved in breeding programs of citrus rootstocks. The use of newly generated codominant markers, SSRs, has allowed a study of colinearity among genomes and the detection of factors forcing heterozygosis. The results constitute a first comparative mapping analysis within the group of true citrus fruit trees.

Materials and methods

Plant material

Three segregating populations were used: family Pp×Pp, derived by self-pollination from *Poncirus trifoliata* var. *Flying Dragon* (57 trees); family A×Pa, derived from the cross between *Citrus aurantium* var. *Afin Verna* and *P. trifoliata* var. *Flying Dragon* (66 hybrids); family V×Pv, derived from the cross between *Citrus volkameriana* and *P. trifoliata* var. *Rubidoux* (80 hybrids). The families Pp×Pp and V×Pv were studied previously by Mestre et al. (1997) and García et al. (1999), respectively. In the present study we have increased the number of markers typed in these progenies by adding SSRs and IRAPs, and we have remade the genetic maps of their parents.

Molecular markers

Simple sequence repeats

Two strategies have been followed to obtain these markers. Microsatellite screening was carried out using the FINDPATTERNS program, GCG package (Wisconsin Package, version 8.1-OpenVMS) in all citrus sequences included at the GenBank up to October 1999, which was searched for all possible repetitions of di-, tri-, tetra- and pentanucleotides. The sequences with microsatellites were used to design specific primers using the PRIME program, GCG package.

The other strategy was to obtain a library of *P. trifoliata* genomic DNA with small-sized fragments. *P. trifoliata* DNA extracted using a CsCl gradient was digested with the *Tsp* 509I restriction enzyme, of which the 4-pb target is compatible with the *Eco*RI target. Digestion was analyzed by agarose gel electrophoresis, DNA fragments of between 300 pb and 650 pb were cut and DNA was extracted using the Agrose Gel DNA Extraction kit of Boerhinger (Indianapolis, Ind.). This DNA was cloned in the vector Lambda Zap II (Stratagene, La Iolta, calif.) using its *Eco*RI recognition sequence, and the construction was packaged using the Gigapack Gold kit (Stratagene).

In total, approximately 90,000 pfu were screened (80,000 with six probes and 10,000 with ten probes). The probes used were: $(ATA)_8$, $(AAAT)_6$, $(CTTT)_6$, $(TTC)_8$, $(ACT)_8$, $(ATC)_8$, $(AAC)_8$, $(CT)_{10}$, $(CTC)_8$ and $(GCT)_8$, which were labeled with digoxigenin using the end labeling kit Dig Oligonucleotide 3′-End Labeling (Boehringer). For screening, phage plaques were transferred to a nylon membrane (Hybond-N, Amersham), then denaturalized with 0.5 *M* NaOH, 1.5 *M* NaCl and fixed for 2 h at 80 °C. The filters were hybridized using mixtures of different probes in 5× SSPE, 1% SDS at 55 °C, rinsed twice with 2× SSPE, 0.1% SDS at 50 °C and rinsed twice again with 0.5× SSPE, 0.1% SDS also at 50 °C. The hybridization signal was subsequently detected using CSPD. All positives were screened twice to reduce false positives and then, the positive clones were converted in pBluescript plasmids by excision in vivo (following the Stratagene protocol). These clones were sequenced, and the PRIME program was used to design specific primers.

The SSRs obtained from the WWW (with the prefix CR), those obtained from the DNA library (with the prefix CL) and those described by Kijas et al. (1997) were amplified and analyzed as in Ruiz et al. (2000). The primers are in the process of being patented and will be commercialized by IVIA.

Inter-retrotransposon amplified polymorphisms

Eight primers were designed from four citrus sequences having homology with the retrotranscriptase domain of the *Copya* family: the sequences were CL3, CL5 and CL6 from *Citrus clementina*, and SI4 from *C. sinensis* (EMBL database accession numbers: CCL131363, CCL131362, CCL131364 and CSI131367). The primers were denoted R or F (reverse or forward) for each sequence and used, alone or in pairs, to amplify intertransposonic sequences. IRAPs were amplified and analyzed as in Bretó et al. (2002).

Within the A×P family, as well as SSRs and IRAPs, some RAPDs, SCARs and resistance analogues (Mago et al. 1999) were also analyzed as in García et al. (1999).

Linkage analysis

JOINMAP 2.0 (Stam 1993; Stam and Van Ooijen 1995) with a linkage criterium of LOD 6, in general, recombination fraction of 0.5 and Kosambi mapping function was used for linkage analysis. The population was analyzed as the "Cross pollinator" population type with no previous knowledge of the linkage phase of the markers. A study was also made of linkage groups obtained by lowering the LOD = 6.0, at intervals of 0.5 units, while maintaining marker order within each group.

Linkage groups with distorted segregation ratios were confirmed and more markers were included using a chi-square test (Mather 1957) for the independence of two segregations, conditional on their marginal frequencies ($\alpha = 1\%$).

The nomenclature used for the linkage groups is as follows. The first letter indicates which parent it belongs to: V (*C. volkameriana*), A (*C. aurantium*) and P (*P. trifoliata*). In the *P. trifoliata* maps, each linkage group has two letters; the second indicates the other (female) parent of family: v (family V×Pv), a (family A×Pa) and p (family Pp×Pp). All groups also have a number: if this is a Roman number (I, II, III,…), it indicates that no homology has been found with respect to linkage groups of other maps, if, by contrast, the linkage group is numbered with Arabic numbers, this indicates that this group presents two or more markers that are common to another linkage group of another map.

The MAPMAKER program (Lander et al. 1987) was also used with a LOD = 3.0, to compare the results with the maps created with JOINMAP. The MAPCHART program (Voorrips 2001) was used to draw the linkage maps.

Results

Molecular markers

A total of 1,477 citrus sequences were located in the database, of which 61 contained microsatellites, but specific primers could only be designed to amplify these microsatellites in 27 sequences. The majority of the sequences found in the GenBank were cDNAs sequenced by Hisada et al. (1997) from *C. unshiu* expressed sequence tags. Since these sequences were short (approximately 300 pb), the position of the microsatellite did not allow the design of specific primers for many of them.

After the first DNA library screening, 81 positive clones were picked up, nine of which were confirmed as carrying one microsatellite in a second check screening. These nine clones were sequenced, and primers were designed for six of them.

As an initial test, microsatellites were analyzed using some individuals belonging to the A×Pa family. We observed that for some microsatellites other products of amplification appeared in addition to the product of the expected size and concluded that these corresponded to other loci. We decided not to modify the conditions nor eliminate these microsatellites, given that the extraproducts did not interfere with the main one in the genetic interpretation and contributed more polymorphic markers to be included in the genetic maps.

Table 2 Number and type of marker in each map: P_a, map of *P. trifoliata* belonging to the family A×Pa; A, map of *C. aurantium* belonging to the family A×Pa; P_v, map of *P. trifoliata* belonging to the family V×Pv; V, map of *C. volkameriana* belonging to the family V×Pv; and P_p, map of *P. trifoliata* belonging to the family Pp×Pp

		Markers	Linked markers	Linkage groups	centiMorgans	SSR[a]	IRAP	RAPD	RFLP	Isoen-zymes	Others
A×Pa	P_a	48	40	5	275.2	23 (73.3%)	15	5			5
	A	120	104	15	441.5	54 (84.4%)	53	8			5
V×Pv	P_v	73	43	8	341.9	30 (80.6%)	11	21	6	3	2
	V	97	79	10	460.1	51 (94.3%)	8	22	11	4	1
Pp×Pp	P_p	66	43	10	269.7	29 (79.3%)	26		2	5	4

[a] In parenthesis, the observed heterozygosity calculated for each of the maps using SSRs

Table 3 Number of bands, number of polymorphic bands and heterozygosity values in the family A×Pa for each of the parents and retrotransposon (RT) sequences used in the primer design. Heterozygosity has been estimated as number of polymorphic bands divided by the total number of bands

RT sequence	Parental	Bands	Polymorphisms	Heterozygosity (%)	
CL3	*C. aurantium*	25	12	48.0	33.3
	P. trifoliata	20	3	15.0	
CL5	*C. aurantium*	16	10	62.5	42.9
	P. trifoliata	19	5	26.3	
CL6	*C. aurantium*	22	8	36.4	20.8
	P. trifoliata	26	2	7.7	
SI4	*C. aurantium*	11	3	27.3	26.7
	P. trifoliata	4	1	25.0	
Total	*C. aurantium*	74	33	44.6	30.8
	P. trifoliata	69	11	15.9	

Table 2 shows the number and type of polymorphic marker used to construct the map of each parent. Heterozygosity for microsatellites varied between 73.3% in *P. trifoliata*, in the A×Pa family, and 94.3% in *C. volkameriana*, in the V×Pv family. To obtain these estimates, only the main microsatellite bands have been used, the polymorphism of which has been produced mainly by a change in the number of repetition units (Litt and Lutty 1989; Tautz 1989; Weber and May 1989; Morgante and Olivieri 1993).

Heterozygosity for IRAPs in the A×Pa family is presented in Table 3. It has been calculated using only the combination of primers based on only one retrotransposon, CL3, CL5, CL6 or SI4 (i.e. primer combinations 3F3R, 5F5R, 6F6R and 4F4R, respectively). Similarly, the minimum number of copies of each retotransposon in the genome of each parent species has been estimated by the number of bands (Table 3). This varies between four for the retrotransposon SI4 in *P. trifoliata* and 26 for CL6 also in *P. trifoliata*.

Linkage maps

Both in the *C. volkameriana* × *P. trifoliata* family and in the *C. aurantium* × *P. trifoliata* family, the parental *Citrus* species are heterozygous at more loci than *P. trifoliata*, making marker density in the *Citrus* spp. maps greater than any *P. trifoliata* map.

Marker segregation distortion was observed for all parents. This distortion was calculated by the JMSLA module in JOINMAP. Segregation distortion affects 23.3% of *C. aurantium* markers and 22.9% of *P. trifoliata* markers in the A×Pa family. This percentage increases up to 39.4 for *P. trifoliata* in the Pp×Pp family. In the V×Pv family, *C. volkameriana* presents distortion for 28.9% of

Table 4 Type of segregation displayed by the different codominant markers in the family Pp×Pp. The expected zygotic frequencies have been calculated using the observed gametal frequencies, for this reason only 1 *df* is used

Linkage group	Marker	Gametal segregation			Zygotic segregation		
		Expected a:b	Observed a:b	χ^2 (1 *df*)	Expected aa:ab:bb	Observed aa:ab:bb	χ^2 (1 *df*)
Pp 3	gp47	51:51	33:69	12.71**	5.3:22.3:23.3	3:27:21	2.24
	TAA 27	57:57	78:36	15.47 **	26.7:24.6:5.7	26:26:5	0.17
Pp 4	CL 1.40_190	40:40	53:27	8.45 **	17.6:17.9:4.6	18:17:5	0.10
Pp 5	TAA 41	56:56	65:47	2.89	18.9:27.3:9.9	15:35:6	4.49*
	CR 25	55:55	60:50	0.91	16.4:27.3:11.4	12:36:7	5.63*
Pp 7	pg 52	54:54	68:40	7.26 **	21.4:25.2:7.4	19:30:5	1.97
	CR 7	56:56	41:71	8.04 **	7.5:26.0:22.5	4:33:19	4.07*
	CR 17	55:55	69:41	7.13 **	21.6:25.7:7.6	18:33:4	4.41*
	CR 18	55:55	69:41	7.13 **	21.6:25.7:7.6	18:33:4	4.41*
Unlinked	cG18	49:49	35:63	8.00 **	6.3:22.5:20.3	6:23:20	0.02

$*P \leq 0.05$, $**P \leq 0.005$

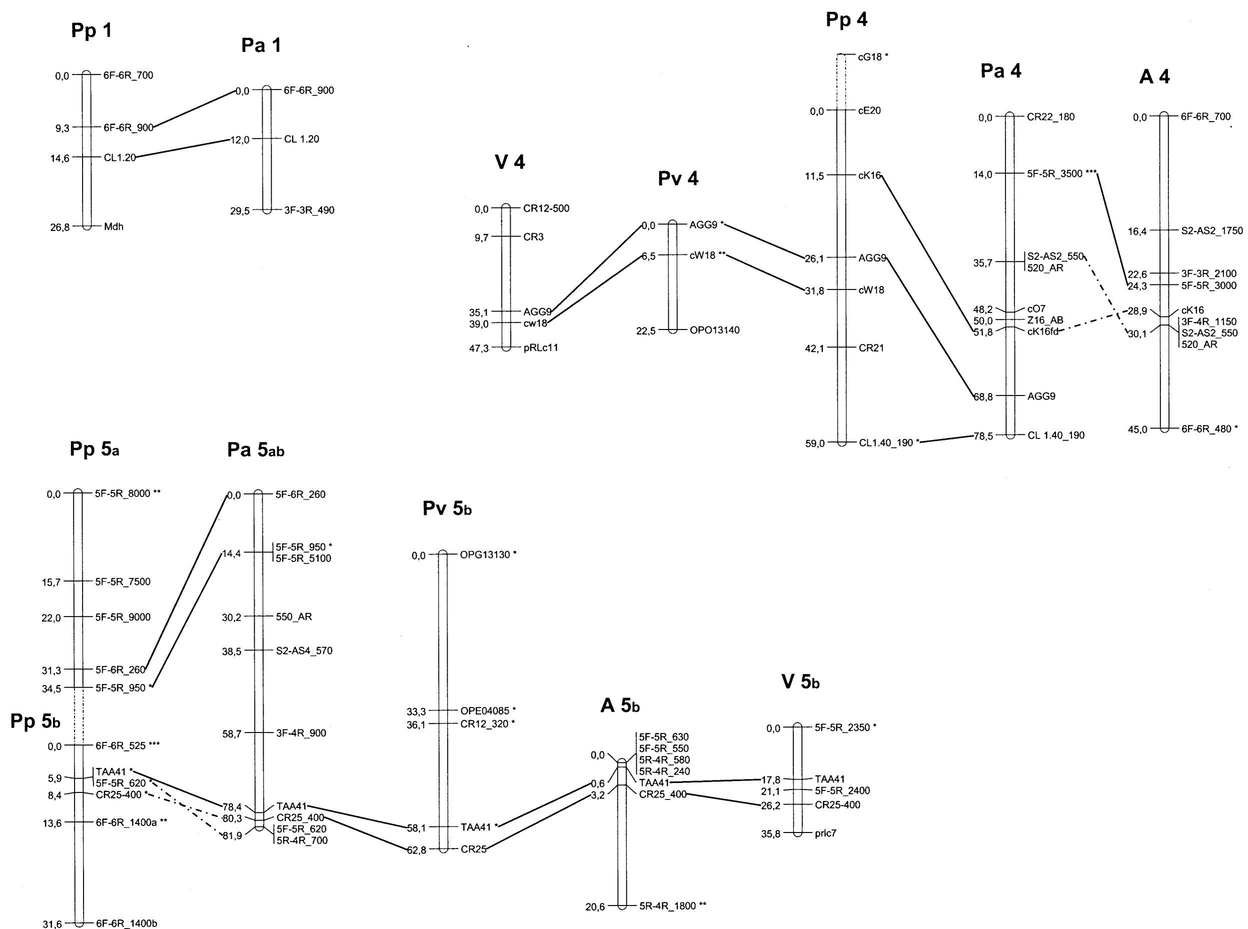

Fig. 1 Linkage maps obtained for each of the parents: *P. trifoliata* var. *Flying Dragon* (*Pp*) from the family Pp×Pp, *P. trifoliata* var. *Flying Dragon* (*Pa*) from the family A×Pa, *P. trifoliata* var. *Rubidoux* (*Pv*) from the family V×Pv, *Citrus aurantium* (*A*) from the family A×Pa, *C. volkameriana* (*V*) from the family V×Pv. Common markers are connected by *lines*; if the order of marker has changed a *discontinuous line* is drawn. *Dotted lines* join positions where a marker has changed of linkage group. *Framed* linkage groups are those of each species where no common markers with any other linkage group has been found

markers, while *P. trifoliata* var. *Rubidoux* presents distortion in 39.7% of the markers. In the P×P family, the two possible levels of distortion, gametal and zygotic, could be investigated. The zygotic frequencies were calculated using the observed gametal frequencies. As shown in Table 4, the linkage groups P_P 3, P_P 4 and the unlinked marker cG18 present a distortion of the gametal segregation. Markers at group P_P 5 have zygotic distortion and those at P_P 7 display significant differences with respect to both gametal and zygotic segregations. Both in P_P 7 and P_P 5b, the zygotic distortion arises from the low number of homozygous individuals observed contrary to expected.

The genetic maps of the parents of each family and a comparison of colinearity within some homeologous *linkage groups are presented in Fig. 1. Unlinked markers are listed in* Table 5.

Discussion

Molecular markers

When the two methods used to develop microsatellites were compared, we found that screening on the WWW was less time-consuming and less demanding on local resources. Using this method we developed 27 new SSRs. The same method has been used in other plant species such as *Arabidopsis thaliana*, maize and sorghum (Senior and Heun 1993; Brown et al. 1996). The efficacy of this method greatly depends on the number of sequences present in the data base of the species under analysis. For example, only two microsatellites were obtained for sorghum because there were only 45 sequences in the data base. The cDNA sequencing project by Hisada et al. (1997) has increased considerably the number of citrus sequences in the data base in recent years. Results from genome projects now in progress will certainly increase the number of SRR markers.

Microsatellite alleles may display the same size but consist of different nucleotide sequences; this is called homoplasy (Estoup et al. 1995; Angers and Benatchez 1997). To get around this lack of information one can use the heteroduplex fragments (Perez et al. 1999), which are two double-chain DNA molecules, each formed by a combination of the two DNA chains corre-

Fig. 1 (continued)

Table 5 List of unlinked markers per parental species

Pp		Pa	Pv		A	V
cG18*	5F-5R_600	CL 2.26	CL 1.20*	CL 1.40–1000	CL 2.26	pRLc39
pgi	5F-5R_2200	TAA27	3F-3R_1000	5F-5R_800	TAA1	OPO13110
Got 1	5F-5R_225***	TAA15	5R-4R_1200	5R-4R_700	CR5_1000***	OPK16120
Got 2	6F-6R_1100	TAA52	6F-6R_900	C1	CR10	OPG13190
CR5	6F-6R_520	k16_800	CR8***	CR19***	K16_1640	OPF14120**
TAA52	5F-6R_2100	CR27_140	CR22-200	CR27_150*	K16_2200	OPF14040**
CR8***	6F-5R_540***	CR16_800*	*Got 2*	OPD07085***	3F-4R_850	OPD07086
CL1.2*	CR20	CR12_520	OPD07087***	OPB5III***	3F-4R_1250	CR16-1050
CR16	CR22-180*		OPE04175	OPG09060**	4F-4R_800	CR12-400
CR26**	CR12-750		OPG09130	OPG13065	6F-6R_660*	CR12-1300*
			OPG13090*	OPG19400	6F-6R_800	cERSx
			OPO13150***	OPO13160	CR12_950	5R-4R_430
			pgi	pRLc103*	CL 1.40_700***	5R-4R_395
			pRLc31	TAA1	CR19_380	C1
			TAA15**		CR19_220	5F-4R_2500***

$^{*}P \leq 0.05$, $^{**}P \leq 0.005$, $^{***}P \leq 0.001$

sponding to the two different alleles of the heterozygous individual. On forming a heteroduplex, regions improperly paired are produced which makes the molecule migrate more slowly on an electrophoresis gel (White et al. 1992). The presence of a heteroduplex allows the differentiation of two or more alleles that have the same electrophoretic mobility (Fig. 2) given that, for each combination of alleles, a different heteroduplex pair is produced. Therefore, the genotype of the individual can be safely assigned.

As well as microsatellites, some sequence-characterized amplified regions, restriction fragment length poly-

Fig. 1 (continued)

morphisms (RFLPs) and isoenzymes have been used to compare linkage maps. Some IRAPs have also been used for this purpose, given that in some cases, with respect to band size and relative intensity, one can determine whether two bands of different families correspond to the same locus. IRAPs are good markers to saturate linkage maps because with a few primer combinations one can obtain many markers with high repeatability and more or less random distribution.

Most IRAPs have been analyzed in the A×Pa family, and the *C. aurantium* map is the most saturated by this marker type. In this map, as in other plant species maps, the retrotransposons are distributed throughout the genome (Brandes et al. 1997; Waugh et al. 1997). However, some zones seem to be enriched. These regions might be hot insertion points (Ananiev et al. 1998). In the remaining maps, although marker density is lower, this distribution pattern can also be observed.

To estimate the minimum number of existing copies of each of the retrotransposons, in the A×Pa family we counted the number of bands that were amplified when direct and reverse primers of the same RT sequence were combined in a polymerase chain reaction. This number

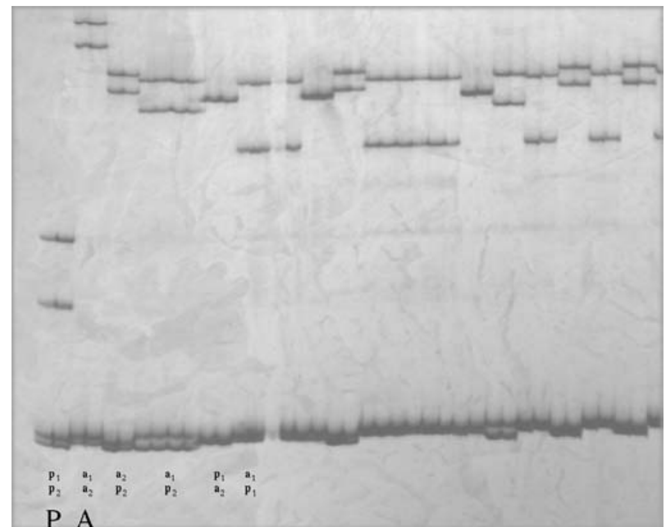

Fig. 2 Silver-stained gel electrophoresis of the SSR marker CR 25 of A×Pa progeny: a_1 and a_2 correspond to the alleles of *C. aurantium* (*A*), and p_1 and p_2 are the alleles of *P. trifoliata* (*P*). The four possible genotypes are indicated by the different combinations of heteroduplex bands (*upper bands*)

could be underestimated because various amplifications might have the same size or because retrotransposon copies exist that are isolated in the genome and are not far enough from each other to permit adequate amplification. On the other hand, one could also think the number is overestimated, seeing that there are various copies in tandem, the number of amplifications is greater than the number of retrotransposon copies; however, if we focus on where the polymorphic bands map (IRAPs), this is not the most frequent case, although tandem copies must exist.

The heterozygosity calculated with the microsatellites is specific to this type of sequence, given that they evolve much more quickly than the rest of the genome (Russell et al. 1997) in the presence of sexual reproduction (Bretó et al. 2002). *P. trifoliata* has lower heterozygosity, between 73.3% and 80.6%, than *Citrus* species, which have values of between 84.4% and 94.3%. The average heterozygosity for the IRAPs is between 44.6% for *C. aurantium* and 16% for *P. trifoliata* (Table 3). That is to say, quite a lot lower than the heterozygosity found for SSRs, even though it follows the same trend – i.e. greater in *C. aurantium* than in *P. trifoliata*.

For the two primers used that correspond to the CL5 sequence, both *C. aurantium* and *P. trifoliata* give a maximum percentage of heterozygosity – 62.5% and 26.3%, respectively. Specifically, a large part of the polymorphisms observed within the species *C. clementina*, closely related with *C. aurantium* (Herrero et al. 1996), corresponds to IRAPs based on this CL5 sequence (Bretó et al. 2001).

Most species of the orange subfamily display high heterozygosity (Herrero et al. 1996). The present results are the first indication that zygotic segregation distortions against homozygotes occurs in one of these species. Since both homozygote classes are reduced in number of individuals, two possible explanations are suggested: the presence of balanced, recessive lethal factors or the presence of gametal incompatibility genes in those genomic regions. These factors, which favor heterozygosity, would explain, at least in part, the high heterozygosity found within the orange subfamily.

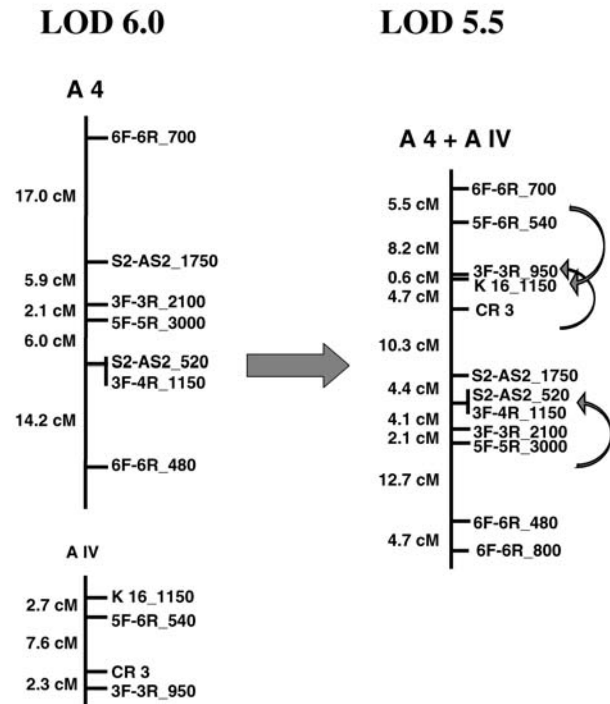

Fig. 3 Linkage groups A 4 and A IV of *C. aurantium*, obtained at LOD = 6.0, which merge on lowering LOD to 5.5. *Arrows*. The site at which the changes in marker ordering took place

Construction and comparison of linkage maps

The genome size of citrus was estimated using the MAPMAKER program (Lander et al. 1987) to be between 1,500 cM and 1,700 cM (Jarrell et al. 1992). If this was true, our maps, made using JOINMAP would cover between 27% and 30.6% of the *C. volkameriana* map and between 16% and 18% of the *P. trifoliata* map in the P×P family.

MAPMAKER is not intended for data with mixed segregation phases, thus it is not a suitable program to analyze these families. Despite this, we used it with the A×Pa family to compare results using JOINMAP. When MAPMAKER was used, closely linked markers, differing in linkage phase, appeared in two different groups. We also observed that while the order of markers was the same linkage groups were 25% longer, on average, when MAPMAKER was used. This coincides with what Mestre et al. (1997) found for the linkage group P_P 4 belonging to the Pp×Pp family. According to Cai et al. (1994) the total size of a linkage group can be reduced by at least 50% using JOINMAP instead of MAPMAKER; thus the genome coverage we have achieved must be underestimated since linkage maps were obtained using the JOINMAP program.

Differences in the distances between two markers in different maps are sometimes remarkable. Differences in the distance might be due to deletions, differences in recombination fraction or just to the sample of individuals. With respect to the applicability of genetic maps on quantitative trait locus analysis and MAS, differences in the order of markers have worse consequences than differences in their distances.

A factor that may contribute to differences in the colinearity between maps is a difference in the LOD criteria. We observed a change in the order of the markers upon lowering the LOD score (Fig. 3). This effect is mostly found when markers are closely linked (and the family size is small) and/or linked markers present segregation distortions. Given that for future genetic analyses, correct ordering is of great importance, we preferred to construct maps at high LOD scores, even though this means that fewer markers fall within the linkage groups and therefore the total length of the genome covered in centiMorgans is apparently lower.

Upon comparing both maps of *P. trifoliata* var. *Flying Dragon*, Pp and Pa (Fig. 1), we found that differences mostly arise from the fact that a few codominant markers were not analyzed in all families. Also, the absence of one marker in a linkage group may prevent the next marker from joining this group, given that the distance may be too large for the next marker to join the group at LOD 6, leaving it, therefore, unlinked.

On comparing the maps of the two varieties of *Poncirus trifoliata*, *Flying Dragon* and *Rubidoux*, we observed a possible reorganization in the map of *Flying Dragon* (Pa) in group 2, where markers CR 19 and TAA 1 are linked at a distance of between 7.1 cM (Pa 2) and 8.7 (Pp 2) cM, while in *Rubidoux* these two markers remain unlinked. There are two possible explanations: (1) a translocation affecting one of these two markers; (2) a paracentric inversion involving both markers. If *Flying Dragon* is heterozygote for this paracentric inversion, the recombination fraction between them would be apparently very low because gametes carrying the recombinant chromatids would not be viable. *Flying Dragon* is a dwarfing rootstock for citrus and according to Cheng and Roose (1995) this characteristic is controlled by just one dominant gene. These same authors suggest that *Flying Dragon* must have originated as a mutation from a small-flowered, non-dwarfing genotype, like *Rubidoux*, given that in the 40 loci markers (isoenzymes and RFLPs) they analyzed, no difference was found. Despite this similarity in marker genotype their genetic maps differ in group 2. It is possible that this reorganization could be responsible for it being a dwarfing rootstock.

When we compared the maps of *Citrus* with those of *Poncirus* we observed that both *Citrus* (*C. aurantium* and *C. volkamer*) maps have zones that are common and, at the same time, differentiate them from the maps of the genus *Poncirus*; for example the complete groups 6 and 11 and parts of group 7 (from CR 23-520 to CL 1.40-290). This is not due to deletions but to the fact that *P. trifoliata* is homozygous for the majority of the markers of these groups.

Three putative marker translocations affecting homeologous linkage groups 3, 7 and 11 were found (dotted lines in Fig. 1). One involves marker CR 12-1000 and differentiates *P. trifoliata* and *Citrus* maps. This marker is located at group 7c in *P. trifoliata* maps, whereas it is found at group 3 in both *Citrus* maps. The other two putative translocations differentiate the *C. aurantium* map from that of *C. volkameriana*. One involves marker TAA27 that is located at V II (linked to V 11) in the *C. volkameriana* map and at A 3 in the *C. aurantium* map. The other marker, CR18_180, is located at V 11 in the *C. volkameriana* map and at A 7b in the *C. aurantium* map.

Four putative inversions were found for linkage groups Pp5b–Pa5ab, Pa4–A4, V7ab–A7b and V3–A3. Two of them affecting the former and the latter linkage groups involve markers spaced less than 2 cM in both parental maps, suggesting they should be attributed to the sampling error of the progenies.

Another factor contributing to differences in the order of markers is the addition of new markers to a previous map, i.e. when a previously constructed map is enriched with new markers. This would be the situation on comparing the maps obtained in the family V×Pv with those previously published by our team (García et al. 1999). Some of the markers have changed their position within the same linkage group. This has happened at groups V 3 and V I within the *C. volkameriana* map and at groups Pv I and Pv 3 within the *Rubidoux* map. In other words, adding new markers to the maps, which is a habitual practice, can bring about a change in the order of some of the previously mapped markers. If the order of the markers in some regions of the maps is so fragile, one must be cautious interpreting QTLs detected at those regions because subsequent MAS schemes may fail.

We can also compare our maps with some of those published previously by other teams (Table 1). One, by a research team at the University of California, has some common microsatellites, isoenzymes and RFLPs. Three versions of this map have been published following successive updates: Jarrell et al. (1992), Kijas et al. (1997) and Roose et al. (2000). The one by Kijas et al. (1997) is the same as that by Jarrell et al. (1992) but with some extra markers (SSRs), and a marker (gp47) disappears only in group H. Some changes in marker order can also be observed. Three of our linkage groups have at least two markers in common with the map by Kijas et al. (1997). Groups V 8 and Pv 8 having markers TAA 52 and *Got 1* must be the same as group A. These markers are also maintained in the map by Roose et al. (2000). The third linkage group corresponds to group D by Kijas et al. (1997), which has the markers *Idh*, pRLc53, TAA 27 and pRLc11. In our maps, the first two markers are in group V 3, but TAA 27 and pRLc11 are found in different groups in the *C. volkameriana* map, while in *C. aurantium*, TAA 27 locates at group A 3. Markers at homeologous group 3 frequently present segregation distortions (Fig. 1, Table 4). This may affect their location, making it less precise, more variable. Nevertheless, we have found that depending on the Citrus species, *C. aurantium* or *C. volkamer*, TAA27 locates at one linkage group or another, suggesting a change in synteny between both species.

We have discusssed here some factors that affect the ordering of markers and consequently make comparative genomics in citrus difficult. These are the addition of new markers, the chosen LOD criterion, the linkage phases, segregation distortions and chromosomal reorganizations. Genetic linkage maps play a prominent role in many areas of genetics: QTL analysis, map-based cloning of genes, marker-assisted breeding and, recently, comparative genomics. Therefore, tools are urgently required for establishing the quality of the data and the maps produced. Although a novel combination of techniques that establishes posterior intervals to the location of markers has recently been reported by Jansen et al. (2001) no software that provides such tools is as yet available.

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