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Evaluation of inter-simple sequence repeat analysis for mapping in *Citrus* and extension of the genetic linkage map

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Abstract Inter-simple sequence repeat (ISSR) analysis was evaluated for its usefulness in generating markers to extend the genetic linkage map of *Citrus* using a backcross population previously mapped with restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and isozyme markers. ISSR markers were obtained through the simple technique of PCR followed by analysis on agarose gels, using simple sequence repeat (SSR) primers. Optimization of reaction conditions was achieved for 50% of the SSR primers screened, and the primers amplified reproducible polymorphic bands in the parents and progeny of the backcross population. Mendelian segregation of the polymorphic bands was demonstrated, with an insignificant number of skewed loci. Most of the SSR primers produced dominant loci; however co-dominance was observed with loci derived from three primers. A new genetic map was produced by combining the segregation data for the ISSR markers and data for the RFLP, RAPD and isozyme markers from the previous map and creating genetic linkages among all the markers using JoinMap 2.0 mapping software. The new map has an improved distribution of markers along the linkage groups with fewer gaps, and marker order showed partial or complete conservation in the linkage groups. The incorporation of ISSR markers into the genetic linkage map demonstrates that ISSR markers are suitable for genetic mapping in *Citrus*.

Keywords *Citrus grandis* · ISSR · Marker · Microsatellite · *Poncirus trifoliata*

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Introduction

The goal of genetic mapping research is simply to find enough polymorphic marker loci so that any genetic locus can be mapped to a specific region of a specific chromosome, preferably within 'walking' distance. The benefits of such a saturated genetic map far outweigh the investment of time and money, especially when combined with a knowledge of traits controlled by loci that can be localized on the chromosomes using available linkage information. The current and most saturated citrus genetic map (Cai et al. 1994) consists of random amplified polymorphic DNA (RAPD), restriction fragment length polymoprhism (RFLP), and isozyme markers and spans 1192 cM, an estimated 70–80% coverage of the genome. With an average distance of 7.5 cM between marker loci, the level of coverage in this map is relatively good for a fruit tree crop. However, an increased number of polymorphic markers would be a distinct advantage for marker-assisted selection and for cloning QTLs (quantitative trait loci) and other loci responsible for traits of horticultural importance. Further saturation of the map is especially desirable to resolve ambiguities in the correlation of cytogenetic evidence and linkage-group characteristics. The linkage groups of the current map are not all uniform in length and marker saturation, whereas the cytogenetic evidence suggests that the chromosomes of citrus are similar in length or show a continuous gradation in size (Sharma and Bal 1957; Guerra 1993), unlike the conspicuous differences in size observed in the current map. It is also important to find co-dominant markers, which are more informative since they can distinguish between heterozygous and homozygous genotypes. These markers can be used as anchors to combine linkage maps produced in different laboratories or with different populations (Kijas et al. 1997) to generate a more comprehensive genetic linkage representation.

RFLPs are by far the largest class of co-dominant markers in plants. Microsatellite markers are now being developed in some plant species including citrus (Kijas

et al. 1997), but progress is slow. These and most other co-dominant markers presently being generated are sequence-based, and in *Citrus*, sequence information is limited, although sequencing efforts are underway (Moriguchi et al. 1998). RFLP markers do not require sequence information; however, the technique of RFLP analysis is laborious and time-consuming, and usually requires the use of radioactive isotopes. Since RAPD markers are primarily dominant, and had already been used to map this population, it was desirable to seek a marker type that would potentially reveal co-dominant loci in the population used for mapping, without the requirement for a priori sequence information. It was also the objective of this research project to accomplish this task as simply as possible.

Therefore, a novel technique described as inter-simple sequence repeat (ISSR) analysis was investigated for its potential usefulness in revealing polymorphisms that could be used as markers, preferably co-dominant, to extend the existing genetic linkage map in *Citrus* (Cai et al. 1994). In this technique, single simple sequence repeat (SSR) primers, which are short oligonucleotides composed of short nucleotide repeats such as are found in microsatellites (Litt and Luty 1989), are used in the polymerase chain reaction (PCR) (Gupta et al. 1994; Wu et al. 1994; Wu and Tanksley 1993). In the first report of ISSR analysis (Zietkiewicz et al. 1994), $(CA)_n$ primers, with or without 5′- or 3′-anchors, were used to reveal multiple loci, presumably microsatellites of varying lengths and genomic locations. ISSR analysis is easy to adopt since primers can be derived from existing literature on SSRs in plants, as well as from sequence database information. The primers do not need to be locus-specific since they will target any region of the genome that contains a complementary microsatellite motif. ISSR markers could in theory segregate as co-dominant markers since they are derived from microsatellite sequences that display co-dominance when analyzed as sequence tagged sites or as random amplified microsatellite polymorphisms (RAMPs) (Wu et al. 1994). They could also map in different regions of the linkage groups than RAPD markers because of the nature of the primers and the sequences they target, which would make them valuable in the extension of the linkage map. ISSR markers have been used to fingerprint trifoliate orange germplasm (Fang et al. 1997) and to identify closely related citrus cultivars (Fang and Roose 1997). For lemon and citrange, 80% or more cultivars were uniquely identified using ISSR markers and 42% of the sweet orange cultivars were distinguishable. This is remarkable since sweet orange cultivars are not distinguishable with any other molecular markers tested (Moore laboratory, unpublished; Gmitter, personal communication).

The previous procedure established for ISSR-PCR analysis in *Citrus* involved the use of radioisotope and polyacrylamide sequencing gels. The merit of ISSR analysis as applied to this research project was its simplicity; therefore, the technique was applied with minimum input, using agarose gels and non-radioactive detection, a deviation from the normal practice of most studies that employ microsatellite-based markers. The reactions were optimized with modifications of the standard laboratory protocol for PCR conditions and the PCR thermal-cycling profile (Cai et al. 1994). The modifications of reaction components that were tested included the titration of magnesium chloride ($MgCl_2$) concentration and the addition of other reagents.

Materials and methods

Plant material and DNA extraction

Sixty progeny plants were available from the BC_1 population: [*Citrus grandis* (L.) Osb.×(*C. grandis*×*Poncirus trifoliata* (L.) Raf.] Durham et al. 1992. Mature, unhardened leaves were selected from plants maintained in the greenhouse, cleaned with moist paper towels, and flash-frozen with liquid nitrogen. The tissue was stored at –80°C. Genomic DNA was extracted with a modified version of the rapid method (Deng et al. 1996). A portion of the DNA was diluted to 2 ng/µl, for use in ISSR analysis, and both the stock and diluted portions were stored at –20°C.

Primers

Primers were purchased in lyophilized form from the University of British Columbia (UBC Set#9) and from commercial custom-primer suppliers, DNAgency, Genosys (Fisher), and Gibco BRL, to replace depleted stock. Additional primer sequences derived from a published work (Fang and Roose 1997) and from scanning *Citrus* cDNA sequences in the GenBank database were also obtained from commercial suppliers. Primer concentration in the reaction mix was 4.4×10^{-4} nmol/µl (0.011 nmol in 25µl). This concentration was adjusted for some primers to improve PCR-product formation. A list of the primers screened is available on the web (Sankar and Moore 1999).

PCR conditions

PCR was carried out in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) as described by Cai et al. (1994) with modifications of the annealing temperature to optimize the reaction conditions for individual primers. Double-stringency profiles (Matioli and de Brito 1995) composed of two series of cycles at two different annealing temperatures were also tested for some primers. Reaction components were put in microplates or microtubes. An overlay of mineral oil (1–2 drops) was used when required to minimize evaporation. The contents of the reaction mix were as follows: 2.5 µl of 10× *Taq* buffer (Mg-free, Promega), 1.0 µl of dNTPs (20 mM), 2.0/1.5 µl of $MgCl_2$ (25 mM), 1.0 µl of primer (0.011 nmol/µl), 7.5 µl of DNA template (2 ng/µl), 0.2 µl of *Taq* polymerase (Promega, 5 U/µl), sterile H_2O, to 25 µl. Negative controls were used in all experiments with sterile H_2O to replace the DNA template. Some reactions were done using RED*Taq* polymerase (Sigma) and its accompanying buffer. For these reactions, the final reaction volume was 50 µl and all reagent amounts were adjusted to maintain consistency in reaction composition except when attempting to optimize the $MgCl_2$ concentration. Other components added to test for improvement in the quality of banding patterns included 10 mM ammonium sulfate and 2% formamide.

SSR primers were screened with a subset of the BC_1 population, and the reaction conditions were modified to improve the banding patterns obtained for each primer. Primer optimization was determined by visual assessment of the banding patterns for intensity, polymorphism and repeatability. The optimum conditions chosen for each SSR primer were based on the best combina-

tion of those three parameters, with special emphasis on repeatability of the reactions. Repeatability was determined between experiments and between parental and a few progeny samples within experiments.

Gel electrophoresis

PCR products (5 or 8 µl) were separated by electrophoresis in TAE buffer at 60 V for about 2.5 h, or at 50 V for 3 h, through a mini-wide gel composed of 1% each of Synergel (Diversified Biotech) and agarose (Gibco), or 1.5% Synergel (Perlman 1991) and 1% agarose. Gels were stained with ethidium bromide (2 µg/ml) and visualized with a UV transilluminator. Gel pictures were recorded using the IS-1000 Digital Imaging System (Alpha Innotech Corporation).

Band scoring and test for random segregation of loci

The BC_1 population was genotyped using the optimized SSR primers and polymorphic bands determined to be consistently repeatable were scored for their presence or absence in parental and progeny samples in the gel profile. The segregation ratios observed among the progeny for putative ISSR markers were tested for goodness of fit to hypothesized Mendelian ratios using the chi-square test. The values were verified by comparing them to the results obtained with the single locus analysis module of JoinMap (JMSLA) version 2.0 (Stam and van Ooijen 1995).

Mapping

Scored data for the ISSR markers were formatted for the JoinMap 2.0 program and analyzed in concert with genotype data for the markers of the current linkage map to produce a new genetic map that incorporated the ISSR markers into the existing linkage groups. Linkages were established using an initial log-likelihood of the odds (LOD) score of 3.0, followed by a lower LOD score to add the rest of the markers. Recombination frequencies between pairs of markers in each resulting linkage group were calculated and the assembled linkage groups were ordered with the JoinMap mapping module (JMMAP) using the Kosambi mapping function. The final linkage map was drawn using DrawMap (Stam 1993).

Results and discussion

Optimization

Optimum conditions were established for 45 SSR primers. Primers that were not optimized produced either little or no PCR product, or could not amplify reproducible or polymorphic banding patterns. Very few primers produced optimal results at the same annealing temperature, even when the theoretical annealing temperatures (T_d) were calculated to be equivalent. There were also no clear trends in the requirement for ammonium ions (NH_4^+), or for a specific concentration of magnesium chloride ($MgCl_2$). Table 1 lists the optimum conditions established for some of the optimized primers.

PCR conditions

Primers were screened initially by titrating the concentration of $MgCl_2$ in the reaction mix. Formamide addition was tested but did not improve the efficiency of PCR product formation with any of the primers, so testing was discontinued. Addition of NH_4^+ proved to be effective in the improvement of PCR-amplification for some primers. The screening routine that prevailed in later experiments was at two different concentrations of $MgCl_2$ with and without NH_4^+, using a subset of the population. Several primers were successfully optimized using a simple annealing temperature modification in the profile of Cai et al. (1994) combined with the reagent modifications. However, for other primers the double-stringency profile was needed to produce optimum results.

Trends in sequence type

Table 2 lists sequences of the primers tested and optimized, with details of the sequence type and presence or absence of anchors. Dinucleotide primers containing the bases A and T were not very easily optimized. Greater than 50% optimization was obtained for all but one of the other combinations tested and more than 70% of (C, T) and (A, C) dinucleotide primers were optimized. This trend in sequence content was previously observed (Gupta et al. 1994; Fang and Roose 1997) although Fang and Roose (1997) did not obtain amplification with dinucleotide $(TC)_n$ primers in addition to $(AT)_n$ primers. They suggested that the apparent lack of amplification observed with (A, T) dinucleotides may be due to self-complementarity within the primer. Their hypothesis would explain the disparity of these observations with the conclusion (Wang et al. 1994) from EMBL and GenBank database surveys that $(AT)_n$ is the most abundant repeat in plants. Trinucleotide optimization was in general more successful than dinucleotide optimization, since 75% were optimized compared to 56% of the dinucleotides screened. Only one tetranucleotide of the six tetra- and penta-nucleotides screened was optimized.

Observations of the PCR products obtained using the eight 5′-anchored primers surveyed in this study suggest that optimized 5′-anchored primers amplify fewer bands than 3′-anchored primers. Fang and Roose (1997) found that the few 5′-anchored primers they tested produced fewer fragments that were generally of larger size compared to those produced by 3′-anchored primers. However, it is difficult to conclude definitively on this without further testing of more 5′-anchored primers to match the number of 3′-anchored primers. Most research efforts to date have not reported on the comparative efficiency of 5′- and 3′-anchored primers in PCR. Zietkiewicz et al. (1994) reported a broader specificity obtained with 5′-anchored primers than the 3′-anchored primers surveyed. The 5′-anchored primers gave a higher density of poorly resolved PCR products in animal DNA samples and gave clear patterns with templates that had a low $(CA)_n$ content. However, as only $(CA)_n$ and $(TG)_n$ repeats were assessed, the observations may only be valid for those repeat motifs. The differences between 5′- and

Table 1 Conditions for optimal amplification of primers and derived ISSR markers

Primer	Annealing temperature (°C)	NH_4^+ [b] /10 mM	$MgCl_2$ (mM)	Total bands	Stable polymorphic bands	Putative loci
807 $(AG)_8T$	50	–	1.5	4	2	–
809 $(AG)_8G$	56..48[a]	+	1.5	6	2	2
810 $(GA)_8T$	55	+	2.0	3	1	1
811 $(GA)_8C$	48	–	1.5	7	2	2
812 $(GA)_8A$	52	–	2.0	6	3	3
814 $(CT)_8A$	52..50	+	2.0	6	3	3
815 $(CT)_8G$	42	–	1.5	8	2	2
816 $(CA)_8T$	55	+	1.5	9	3	3
817 $(CA)_8A$	52..50	+	2.0	7	3	3
818 $(CA)_8G$	46..35	–	1.5	9	3	3
819 $(GT)_8A$	48..35	–	1.5	4	1	1
820 $(GT)_8C$	48..35	+	1.5	2	1	1
821 $(GT)_8T$	45..35	–	1.5	4	2	2
822 $(TC)_8A$	52	+	2.0	2	1	1
823 $(TC)_8C$	55	–	2.0	6	1	1
824 $(TC)_8G$	55	–	2.0	4	2	2
825 $(AC)_8T$	52..35	–	1.5	7	3	3
826 $(AC)_8C$	52..50	–	1.5	7	2	2
827 $(AC)_8G$	56..50	–	2.0	9	5	5
828 $(TG)_8A$	46	+	1.5	4	1	1
830 $(TG)_8G$	46	+	1.5	7	2	2
834 $(AG)_8YT$	48	–	2.0	10	3	3
840 $(GA)_8YT$	52..35	–	2.0	10	1	1
843 $(CT)_8RA$	48	+	1.5	2	1	1
844 $(CT)_8RC$	55	+	2.0	4	1	1
846 $(CA)_8RT$	48	–	2.0	6	1	1
847 $(CA)_8RC$	52..50	+	2.0	7	3	–
849 $(GT)_8YA$	55	–	1.5	10	3	3
851 $(GT)_8YG$	52..35	–	2.0	6	2	2
853 $(TC)_8RT$	42	+	2.0	2	2	2
855 $(AC)_8YT$	52..50	–	1.5	11	1	1
856 $(AC)_8YA$	52..48	+	2.0	6	3	3
857 $(AC)_8YG$	52..48	–	1.5	7	4	4
858 $(TG)_8RT$	48..35	–	1.5	8	3	3
859 $(TG)_8RC$	48..35	–	1.5	10	3	2
861 $(ACC)_6$	55	–	2.0	12	2	2
864 $(ATG)_6$	52	+	2.0	8	3	3
866 $(CTC)_6$	48..35	–	2.0	7	2	2
868 $(GAA)_6$	48	–	1.5	13	2	2
873 $(GACA)_4$	53	–	1.1	6	1	–
ISSR1 $BDB(TCC)_5$	53	–	2.0	5	2	2
ISSR2 $HVH(TCC)_5$	53	–	1.5	4	1	1
ISSR5 $(TCC)_5RY$	53	–	1.5	6	2	2
ISSR7 $(TAA)_8$	42	+	1.5	7	3	3
ISSR8 $(CAC)_6$	52..50	–	1.5	10	1	1

[a] Indicates a double-stringency profile
[b] + indicates where NH_4^+ was required for optimal PCR product formation

3′-anchored primers may also be a function of the number of bases present in the anchor sequence. Most of the 5′ anchors tested contained three bases, while the 3′ anchors were single-base anchors. This may have contributed to differences in amplification. Some differences were evident when comparing dinucleotide primers that differed in the base composition of their anchor sequence. Among the anchored dinucleotides, 29% more of the primers with single-base 3′ anchors were optimized than those anchored with two bases.

Polymorphisms revealed with ISSR-PCR

PCR products obtained with ISSR analysis were similar to the products of RAPD analysis. This is evident from observations of the average number of bands amplified and the size range in kilobases of the fragments produced (Table 1, Fig. 1). Optimized SSR primers amplified 2–13 bands ranging in size from 100 to 3000 bp, with an overall average of six bands amplified. The level of polymorphism was lower than that reported for other ISSR-PCR studies employing more complex gel-electrophoresis techniques to reveal ISSR marker loci (Fang and Roose 1997) and can be explained by the simpler technique applied. The number of stable polymorphic bands obtained ranged from one to five. Differences in the number of stable polymorphic bands and the number of putative markers in Table 1, and the absence of putative markers for some primers, are due to problems encountered in scoring bands that were stable and polymorphic but difficult to score in some progeny. This was due

Table 2 Number and type of SSR primers tested and optimized (bracketed)

Sequence type	Repeat	Anchor type					Number tested (Optimized)		Percent optimized
		None	3′		5′		Sub-total	Total	
			1-base	2-base	3-base	4-base			
A, G	Di-	–	6 (5)	6 (2)	–	–	12 (7)	14 (8)	57
	Tri-	1 (1)	–	–	–	–	1 (1)		
	Penta-	1 (0)	–	–	–	–	1 (0)		
A, T	Di-	–	6 (0)	4 (0)	–	–	10 (0)	11 (1)	9
	Tri-	1 (1)	–	–	–	–	1 (1)		
C, T	Di-	–	6 (5)	5 (3)	–	–	11 (8)	16 (12)	75
	Tri-	1 (1)	–	1 (1)	2 (2)	–	4 (4)		
	Tetra-	1 (0)	–	–	–	–	1 (0)		
G, T	Di-	–	6 (5)	6 (4)	2 (0)	–	14 (9)	16 (9)	56
	Tri-	1 (0)	–	–	–	–	1 (0)		
	Penta-	1 (0)	–	–	–	–	1 (0)		
A, C	Di-	–	6 (6)	7 (5)	3 (0)	1 (0)	17 (11)	19 (13)	72
	Tri-	2 (2)	–	–	–	–	2 (2)		
A, G, T	Tri-	2 (1)	–	–	–	–	2 (1)	4 (1)	25
	Tetra-	2 (0)	–	–	–	–	2 (0)		
A, C, T	Penta-	1 (0)	–	–	–	–	1 (0)	1 (0)	0
A, C, G	Tetra-	1 (1)	–	–	–	–	1 (1)	1 (1)	100

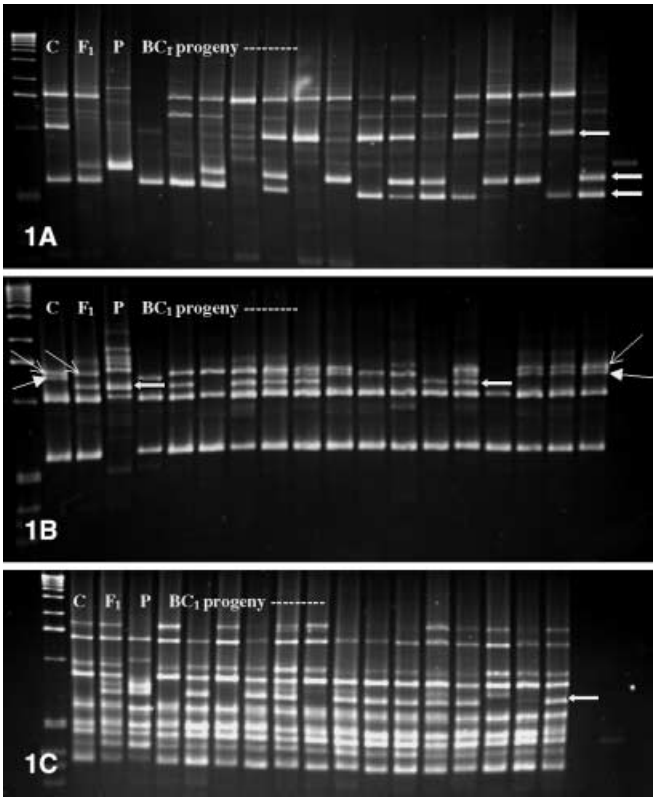

Fig. 1 Gel Profiles of PCR-amplified fragments from ISSR analysis using primers UBC 825 (**A**), UBC 856 (**B**) and UBC 861 (**C**) with the parents (*C*, F_1, *P*) and progeny (BC_1) of the BC_1 population. *Arrows* point to polymorphic bands. In **B**, the *arrow type* indicates which parental band matches the band found in the progeny

Table 3 Summary of observed segregation types

Segregation type	Expected ratio	Number of markers	Number of skewed loci
Aa×Aa	3:1	24	0
Aa×aa	1:1	15	3
aa×Aa	1:1	43	8
1,1×1,2[a]	1:1	3	0

[a] Co-dominant segregation

to overlapping fragments that complicated genotype designation and made it necessary to exclude those bands from consideration as putative marker loci.

ISSR-marker segregation types

The list of putative markers and their hypothesized mode of segregation with the chi-square results is available upon request or on-line (Sankar and Moore 1999). Inspection of the chi-square values for the ISSR markers revealed that most of them displayed normal Mendelian segregation although a few exhibited segregation distortion (about 13%). Most of the skewed loci were of the "aa×Aa" type and were skewed toward the *C. grandis* parent. These results are similar to the findings for markers of the previous map (Cai et al. 1994) for which 73% of the distorted loci were skewed towards *C. grandis*. There are fewer distorted loci among the ISSR markers than among RAPD markers, which may be attributed to the nature of the ISSR markers, or to the smaller number

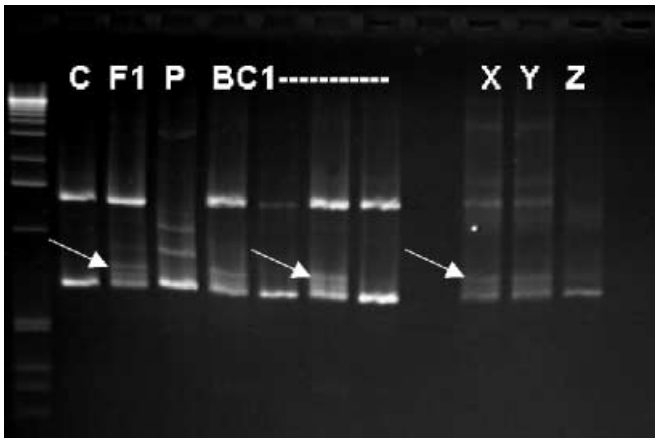

Fig. 2 Heteroduplex bands (*arrows*) in the PCR products of F_1 and BC_1 progeny are generated in the intermixed PCR products (*X*, *Y*) of parents *C* and *P*. *Lane Z*=control. Intermixing was done by mixing 8 µl of the PCR products of each of the parents with 5 mM of EDTA (to inhibit polymerase activity) and re-heating the whole mixture to 94°C, then cooling at room temperature to allow re-annealing (Novy and Vorsa 1996)

of markers surveyed. The number of loci that were distorted and the segregation types observed are summarized in Table 3.

Observations of the ISSR marker segregation types confirm the high level of heterozygosity that was previously reported for the recurrent parent, *C. grandis* (Cai et al. 1994). This apparent trend in heterozygosity was explained by the amplification of sequences outside of coding regions by RAPD primers, in contrast to loci revealed by RFLP analysis (Durham et al. 1992). This hypothesis concurs with the report (Morgante and Olivieri 1993) that microsatellites can also be found outside of coding regions.

Putative ISSR marker loci displayed mainly dominant segregation (96.5%) and co-dominant segregation was only evident with three loci. The three primers that generated co-dominant markers were UBC 834, ISSR 1 and ISSR 2. Co-dominant segregation was hypothesized when the gel profiles of those primers appeared to match the heteroduplex banding reported by Novy and Vorsa (1996). The occurrence of heteroduplex bands explained the appearance of gel profiles in which pairs of fragments were either jointly present or jointly absent in the parents or progeny. Dominant segregation was rejected as a valid possibility when the heteroduplex bands could be generated de novo by inter-mixing the PCR products of the parents (Fig. 2). The inter-mixing of PCR product from parents *C. grandis* and *P. trifoliata* produces a heteroduplex profile that is identical to that from the F_1, although these two parents do not produce heteroduplexes following PCR without inter-mixing their DNA.

Genetic mapping of ISSR markers

Grouping and ordering of the markers

Output from the JoinMap 2.0 grouping module (JMGRP) analysis of the combined genotype data for 85 ISSR markers plus the markers for each linkage group of the existing map was inspected at a LOD score of 3.0. At this LOD score, JMGRP produced a grouping of the markers that, when evaluated, resulted in a linkage map containing nine linkage groups. The recombination frequencies of markers in each group were calculated with the JoinMap recombination module (JMREC). The recombination values from JMREC were used to order the markers in each linkage group with JMMAP. JMMAP used recombination frequency (REC) values and the parameters defined in the response files to detect the best-possible arrangement of all of the markers. The response file parameters were constant for all but four linkage groups, for which effective changes were made in the "lod" value.

A few markers (18%) were excluded from the linkage groups assembled with the JMGRP results in the first round of mapping. These were ISSR and RAPD markers that did not show linkage to any of the major linkage groups at a LOD score of 3.0. The genotype data for these markers was appended to the data for newly formed linkage groups to be re-analyzed by JMGRP. The groupings that resulted enabled a few more markers to be assigned to the linkage groups at LOD scores at or above 3.0. The rest of the markers were assigned to the linkage groups at LOD scores lower than 3.0 but not less than 2.0. Recombination values were re-calculated with JMREC and the resulting linkage groups were again ordered with JMMAP. Several markers were unmapped because they did not show linkage to any other marker at or above the lower LOD score limit of 2.0 set for JMGRP.

Map features

The final version of the map (Fig. 3) consists of nine linkage groups with 310 markers, and spans 874 cM as calculated with the Kosambi mapping function. The total length of the new map is reduced compared to the previously published version, which may be because of the reduction in the overall average centimorgan distance between adjacent loci (3.32 cM), and the smaller number of gaps greater than 20 cM in the new linkage groups. The reduction in the total map length may also be a result of using the JoinMap program, which has been reported to greatly reduce map centimorgans in comparison to the values obtained with MAPMAKER (Cai et al. 1994) which was used to produce the previous map. An extrapolation of the map length, according to the rough conversion deduced from the observations of Cai et al. (1994), puts the new total map length at around 1457 cM, which would in effect be an increase in map length

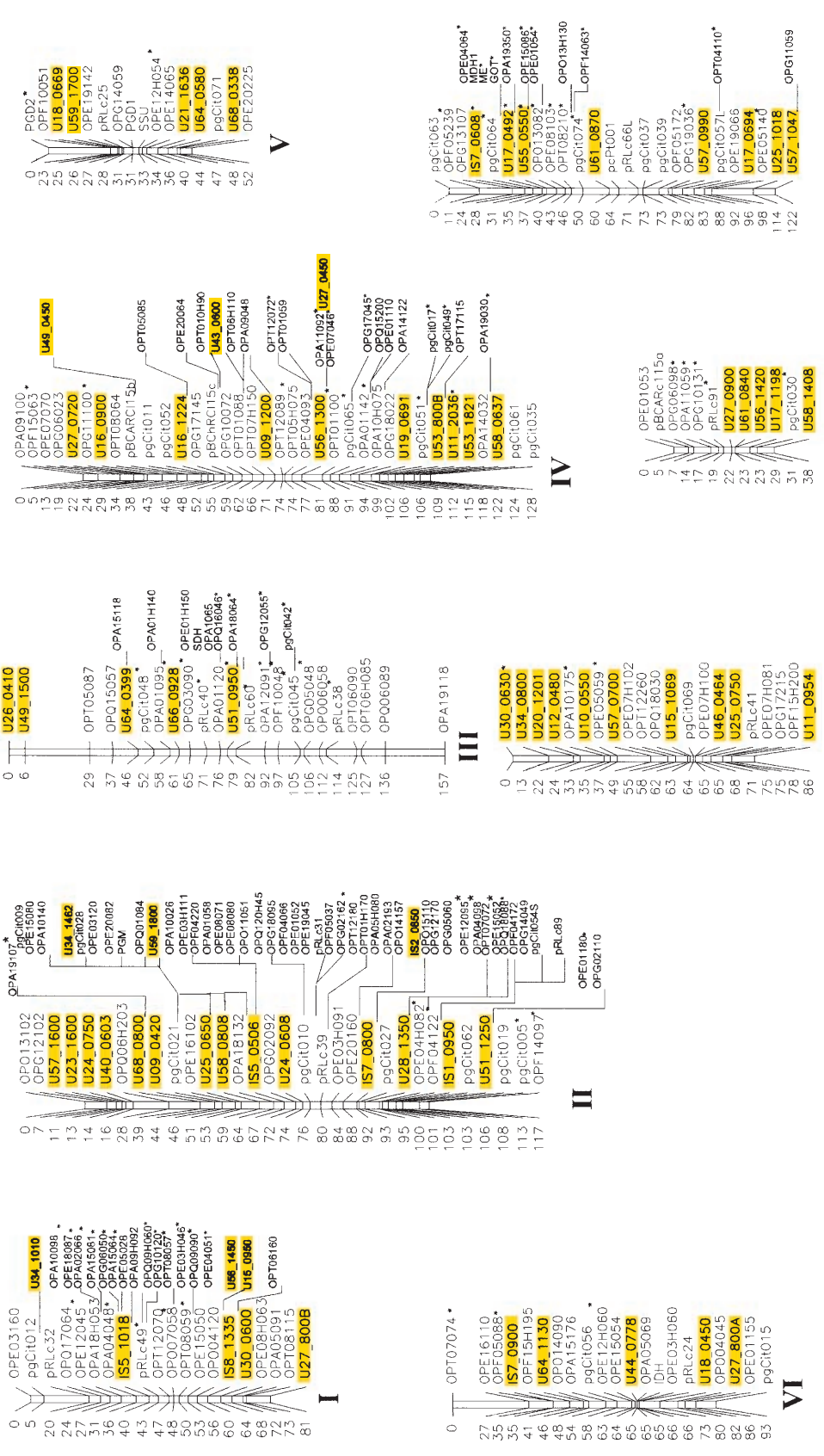

Fig. 3 Citrus genetic linkage map with incorporated ISSR markers. Linkage group numbers correspond to the linkage groups of Cai et al. (1994), except for IX, which was originally part of linkage group I. *Asterisks* indicate skewed loci. ISSR markers are *highlighted*

Table 4 Features of Citrus genetic linkage map

Linkage group	Segregation types			Average distance /cM	Largest gap /cM	Markers	
	aa×Aa	Aa×Aa	Aa×aa			ISSR	Total
I	23	7	8	2.1	15	7	38
II	52	14	12	1.9	12	17	78
III	26	5	1	5.2	23	5	32
IV	41	8	7	2.4	8	13	56
V	14	1	1	3.3	23	5	16
VI	13	5	3	4.4	27	5	21
VII	16	5	0	4.1	13	10	21
VIII	10	1	1	3.2	8	5	12
IX	26	4	6	3.4	16	8	36
Total assigned	221	50	39				
Unassigned	5	6	10				
Total	226	56	49			75	310

from the previous map. The map distance in centimorgans is also affected by the mapping function used in the analysis, and the Kosambi mapping function gives a different estimate of map distance than the Haldane mapping function. For comparison, map distance calculations were done with the Haldane mapping function, using the same parameters as for the Kosambi mapping function, and the resulting map was 1249.2 cM long.

The location of previously mapped markers corresponds to the linkage group numbers of the new map for seven of the linkage groups. The other two linkage groups (LG I and IX) are the result of a split between markers of the previous LG I. Markers that were part of the original LG IX were incorporated into LG III. Linkage groups I, II, III, IV, V and VI are shorter than their counterparts on the previous map. This is expected for LG I since it was split into two groups. However, it is also not unreasonable to expect a reduction in the size of the other linkage groups since the average distance between loci on these linkage groups is much reduced, and only LG VI has a gap larger than 25 cM. The largest gap in LG II and LG IV is now 11.9 and 8 cM, respectively. Group II also has the smallest average distance between loci.

There are 75 ISSR markers dispersed among the linkage groups of the new map (Table 4), with little clustering except for three markers in LG II, which all mapped to the same location. These markers did not display segregation distortion and the primer sequences from which they were generated are unrelated; thus the apparent tight linkage between these markers may possibly be attributed to the close linkage of the corresponding microsatellite loci in the genome of *Citrus*.

Other areas of general clustering among markers corresponded to that observed by Cai et al. (1994) near to one end of LG II, but with additional clustering in other areas of this group, and near the middle of LG VI as in the previous map. Markers were also clustered along the length of LG IV, which has an average distance of 2.4 cM between loci, and in parts of LG VII. Generally, markers that were part of clusters on the previous map were found in the clusters of the new map, but there are some exceptions. In support of the observation that distorted loci tend to cluster in some regions (Cai et al. 1994), distorted ISSR loci in LG IX mapped among a cluster of distorted loci from the previous map. Although the distorted ISSR loci on other linkage groups did not map among previously identified clusters, their map positions were near to other distorted RAPD, RFLP or isozyme loci.

Marker segregation type affected the assignment of markers to the map with conspicuously similar results to that reported previously (Cai et al. 1994). Table 4 displays the distribution of allelic configurations among the linkage groups of the new map. The proportion of unassigned loci in each class observed here are almost identical to that reported by Cai et al. (1994). Among the unassigned markers there are twice as many markers of the "Aa×aa" configuration, and overall there are three-times as many markers for which *C. grandis*, the recurrent parent, is heterozygous.

The marker order of previously mapped markers in the new linkage groups is fairly conserved for linkage groups V, VI, VII and VIII. There is very little conservation in LG II and partial conservation in LG IV, IX and I. The changes in marker order may be due to the incorporation of ISSR markers into the linkage groups which would have affected the statistical estimation of the map distances between the markers, thus altering the relationship between adjacent loci that may not have been strongly linked. Marker order may also have been affected because of the difference in the mapping software used to process the markers of the previous and current map. The smaller linkage groups that contained fewer markers showed a lower tendency to lose the original marker order. This implies that the conservation of marker order may be a function of the number of markers in the linkage groups. Thus, it is probable that the greater the saturation of markers on the linkage group, the greater the discrepancy will be between results obtained using different mapping programs. The lowest conservation rate was obtained with LG II, which is also the linkage group with the most markers, followed closely by LG IV, the second most saturated linkage group.

A total of 75 ISSR markers and 235 of the markers from the previous linkage map were placed on the final version of the map. ISSR markers appear to be suitable for mapping, as evidenced by the successful incorporation of 88% of the putative marker loci into the *Citrus* genetic linkage map. The dispersed distribution of the new markers on the linkage map and the improved overall saturation of markers also illustrate the benefits of ISSR analysis for mapping. The lower level of segregation distortion of ISSRs, compared to the RAPD markers previously generated in the BC_1 population, is a positive feature that makes them a valuable tool. However, the low percentage of co-dominance observed is a disadvantage, especially in the interest of finding markers to combine maps created for different populations. One possible way to increase the percentage of co-dominant markers is by combining SSR and random primers in the same reaction (Wu et al. 1994). Alternatively, ISSR-PCR products can be cloned and sequenced to convert the ISSR markers into sequence-characterized amplified regions (SCARS) to increase the likelihood of detecting co-dominance. This would improve the utility of ISSR analysis as a convenient and useful alternative to more complex molecular marker techniques.

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