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Integration of trinucleotide microsatellites into a linkage map of *Citrus*

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Abstract We report the successful assignment of the first seven microsatellite markers to the *Citrus* RFLP and isozyme map. A total of 14 microsatellite primer pairs were developed and tested for amplification and product-length polymorphism within a population of plants previously used for linkage-map construction. In each case, the successfully assigned microsatellite mapped to the termini of a different linkage group indicating a widespread distribution throughout the genome. Analysis of allele segregation revealed that two of nine microsatellites displayed a significant deviation from expected ratios ($P > 0.5$). This was compared with other marker types within *Citrus* and a similar proportion of skewed loci was also found to be present. The analysis of two markers was complicated by the non-amplification of an inherited null allele within the mapping population. The successful integration of microsatellites into the genetic map of *Citrus* demonstrates the utility of this marker type for genetic analysis within wide intergeneric plant crosses.

Key words *Citrus* · Microsatellites · STS markers · SSLP · SSR · Null alleles

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Introduction

To facilitate the eventual map-based cloning of economic-trait loci the establishment of detailed genetic maps has been undertaken in a wide range of plant species. The development of genetic maps is of special importance for fruit crops such as citrus which have very long generation times and where breeding is complicated by a high level of apomixis (Gmitter et al. 1992). The identification of markers linked to traits such as cold hardiness, shortened juvenile period and resistance to pests such as the citrus tristeza virus, will be invaluable in marker-assisted breeding programs aimed at plant improvement.

To-date, most genetic maps have been based on restriction fragment length polymorphisms (RFLPs), for example in rice (Causse et al. 1994), sorghum (Xu et al. 1994), bean (Nodari et al. 1993), tomato and potato (Tanksley et al. 1992). These are termed type-I markers (O'Brien et al. 1993) and three RFLP-based linkage maps have been produced within citrus. Each was constructed from highly heterozygous intergeneric hybrid crosses which allow a range of segregating characteristics to be genetically dissected. One map contains 46 markers (Jarrell et al. 1992) while the other two, constructed within the same cross, have a total of 62 markers (Durham et al. 1992) and each contain 11 linkage groups. The second of the two maps has recently been extended to include 109 random amplified polymorphic DNA (RAPD) markers, which has successfully condensed the number of linkage groups to equal the haploid chromosome number n = 9 (Cai et al. 1994). This map is estimated to cover 70–80% of the *Citrus* genome and spans a total length of 1192 cM.

The discovery of simple sequence repeats, or microsatellites, has provided the opportunity to significantly increase the density of linkage maps. The highly polymorphic nature of microsatellites has made them the type-II marker of choice used to construct the maps of mouse (Copeland et al. 1993) and human (Weissenbach 1993). PCR-based markers have a number of technical advantages over RFLP analysis; for example, small amounts of DNA are required and the polymorphism is revealed without the need for probe hybridisation. In addition, microsatellite markers have the advantage of showing co-dominant inheritance and, being a high-stringency PCR approach, doses not suffer the problem of reproducibility associated with RAPD profiles (MacPherson et al. 1993; Schierwater

and Ender 1993). Within plants, large-scale mapping initiatives are underway using microsatellites, and the ability to map microsatellites has been demonstrated in rice (Wu and Tanksley 1993), maize (Senior and Heun 1993), soybean (Morgante et al. 1994; Akkaya et al. 1995), barley (Becker and Heun 1995) and *Arabidopsis* (Bell and Ecker 1994).

The goal of the present study was to integrate microsatellite markers into a RFLP map derived from an intergeneric cross made between *Citrus* and the related genus *Poncirus*. The ability, however, to map plant microsatellites in very wide crosses has not been fully investigated and is dependent on sufficient sequence conservation being present within priming sites to amplify homologous loci across the genera of interest. The extent to which microsatellite primers are able to amplify homologous loci between plants seems to vary. Wu and Tanksley (1993) found primer sequence conservation existed among cultivated rice subspecies and related wild species, while Thomas and Scott (1993) found a similar sequence similarity among grapevine; however, Roder et al. (1995), in a study of wheat microsatellites, found very low rates of successful amplification when rye and barley were tested. Previous work within citrus using two microsatellite markers (Kijas et al. 1995) found a high level of sequence conservation between *Citrus*, *Poncirus* and *Severinia*. In the present study we report the testing of 14 microsatellites within an intergeneric mapping population for linkage analysis and map assignment.

Materials and methods

The citrus mapping population

The intergeneric hybrids 'Sacaton' citrumelo (*C. paradisi* Macf. × *P. trifoliata*) and 'Troyer' citrange [*C. sinensis* (L.) Osbeck × *P. trifoliata*] were hybridised to generate the 57 seedlings used for linkage mapping (Jarrell et al. 1992). The cultivars used to represent grandparent lines are *P. trifoliata* 'Pomeroy', *C. paradisi* 'Duncan' and *C. sinensis* 'Olinda valencia'. All the DNA stocks were extracted from plants held in collections at Riverside, California, USA.

Microsatellite enrichment and amplification

A hybrid between rangpur lime (*Citrus* × *limonia* Osbeck) and trifoliate orange [*Poncirus trifoliata* (L.) Raf.] was selected from CSIRO's citrus scion breeding program for DNA isolation and microsatellite development. The DNA was digested with *Mbo*I and ligated into pGEM3-Z (Promega) to create a small-size insert library. This was used as starting material to construct separate libraries enriched for (TAA) and (CAC) microsatellite repeats. This procedure is described in detail elsewhere (Kijas et al. 1994). Following enrichment, libraries were screened and colonies selected and sequenced (see Kijas et al. 1995). Primer pairs were designed with similar theoretical melting temperatures of approximately 60°C. The sequence of each is recorded in Table 1. Oligonucleotides were synthesized commercially (Macromolecular Resources, USA) with the forward primer of each pair being chemically 5′ end labelled with the fluorescent dyes 6-FAM or HEX from Applied Biosystems (ABI). PCR amplifications were conducted in 50 µl containing 50 ng of genomic DNA and 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM total dNTP, 0.5 U *Taq* Polymerase, 0.1 µM of fluorescent forward primer and 0.1 µM of reverse (unlabelled) primer. PCR thermocycling conditions were as follows: initial denaturation at 94°C for 5 min followed by 32 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 1 min. A final 4-min 72°C final extension was included. The primer annealing temperature was lowered to 45°C for 30 s to promote amplification using primer pairs TAA33, TAA52 and CAC23.

Automated microsatellite allele size determination

A 0.3-µl aliquot of each PCR amplification product was mixed with 6 fmol of internal size standard TAMRA GS-500 (ABI). Products from up to three loci were loaded and analysed together, dependent on the allelic size-range of each marker. Sample and standard were heat-denatured before electrophoresis in a 6% denaturing polyacrylamide gel using an automated 373 DNA sequencer (ABI). Fluorescent PCR products were then sized using the GENESCAN 672 software (ABI) and filter set B, meaning that 6-FAM-labelled products appeared blue, HEX-labelled products yellow, and the TAMRA size standard red.

Segregation and linkage analysis

The RFLP and isoenzyme marker segregation was re-analysed along with the microsatellite marker segregation data using JoinMap version 1.4 (Stam 1993). A critical LOD value $\geqslant 2.5$ was used to detect linkage and the Kosambi mapping function (Kosambi 1944) was used to relate map distance and recombination frequency. The linkage group nomenclature employed was that of Jarrell et al. (1992). Loci were tested for segregation distortion using chi-square (χ^2) goodness-of-fit values.

Results

Testing primers within the mapping population

Fourteen primer pairs, developed using a magnetic bead enrichment strategy (Kijas et al. 1994), were tested for amplification and product-length polymorphism within a plant population previously used for linkage-map construction (Jarrell et al. 1992). All 14 primer pairs amplified PCR products from each grandparent, the intergeneric hybrid parent and the F_2 progeny plant genome; however five primer sets generated products unable to be analysed for linkage (Table 1). Not all primer pairs revealed length-polymorphic products within the linkage set, with primers for markers CAC15 and CAC39 amplifying the same length product across all genomes (Table 1). Amplification of a third marker, CAC33, resulted in a PCR-product pattern too complex to analyse and this marker also could not be mapped.

The remaining two markers not tested for linkage had analysis complicated by the presence of non-amplifying alleles (Table 1). Examination of visible allele inheritance within the mapping pedigree, as shown for microsatellite TAA3 in Fig. 1, demonstrates the presence of a non-amplified allele being transmitted

Table 1 Microsatellite mapping and position

Locus	Primer sequence[a]	Linkage group	Comments
TAA1	a-GACAACATCAACAACAGCAAGAGC b-AAGAAGAAGAGCCCCCATTAGC	J	Linked to previously unlinked markers
TAA15	a-GAAAGGGTTACTTGACCAGGC b-CTTCCCAGCTGCACAAGC	I	
TAA27	a-GGATGAAAAATGCTCAAAATG b-TAGTACCCACAGGGAAGAGAGC	D	
TAA33	a-GGTACTGATAGTACTGCGGCG b-GCTAATCGCTACGTCTTCGC	H	
TAA41	a-AGGTCTACATTGGCATTGTC b-ACATGCAGTGCTATAATGAATG		Remains unlinked
TAA45	a-GCACCTTTTATACCTGACTCGG b-TTCAGCATTTGAGTTGGTTACG		Remains unlinked
TAA52	a-GATCTTGACTGAACTTAAAG b-ATGTATTGTGTTGATAACG	A	
CAC23	a-ATCACAATTACTAGCAGCGCC b-TTGCCATTGTAGCATGTTGG	F	
cAGG9	a-AATGCTGAAGATAATCCGCG b-TGCCTTGCTCTCCACTCC	K	Linked to previously unlinked markers
TAA3	a-AGAGAAGAAACATTTGCGGAGC b-GAGATGGGACTTGGTTCATCACG		Null allele prevented analysis
CAC15	a-TAAATCTCCACTCTGCAAAAGC b-GATAGGAAGCGTCGTAGACCC		Monomorphic
CAC19	a-ACAACCTTCAACAAAACCTAGG b-AAGACTTGGTGCGACAGG		Null allele prevented analysis
CAC33	a-GGTGATGCTGCTACTGATGC b-CAATTGTGAATTTGTGATTCCG		Multiple locus amplification
CAC39	a-AGAAGCCATCTCTCTGCTGC b-AATTCAGTCCCATTCCATTCC		Monomorphic

[a] Primer sequence is recorded 5′-3′ and primer 'a' was labelled with the fluorescent tag in each case

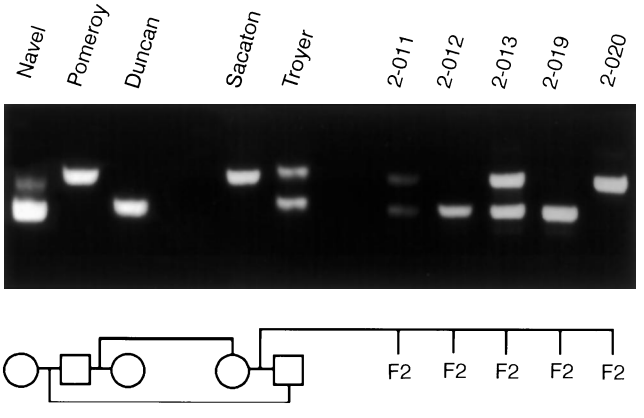

Fig. 1 Polyacrylamide-gel electrophoresis of microsatellite TAA3 alleles amplified within a citrus linkage-mapping population. The relationship of grandparent cultivars 'Pomeroy' (*P. trifoliata*), 'Duncan' (*C. paradisi*) and 'Navel' (*C. sinensis*) to the hybrid parents 'Sacaton' citrumelo (*C. paradisi* Macf. × *P. trifoliata*) and 'Troyer' citrange [*C. sinensis* (L.) Osbeck × *P. trifoliata*] and selected progeny is shown. Visible allele inheritance demonstrates that a segregating non-amplified allele has been transferred from Duncan to Sacaton and then to progeny plants 2-012 and 2-019

from the 'Duncan' grandparent to the 'Sacaton' parent and then to progeny 2-012 and 2-019. This complicates analysis because progeny such as 2-020 (Fig. 1) may be either a homozygote for the upper allele or a heterozygote containing the null allele, and can not be distinguished.

Microsatellite map positions

The segregation information collected in this study from nine microsatellites was analysed along with data from 37 RFLP and eight isozyme markers using the JoinMap program (Stam 1993). JoinMap is able to accommodate data of several segregation types to construct an integrated map containing microsatellite, RFLP, and isozyme marker types. The successful genetic assignment of seven microsatellite markers to the existing RFLP map is illustrated in Fig. 2. The map position of all marker types is shown and all seven microsatellites can be seen to map to the terminus of a different linkage group (Fig. 2 and Table 1). No two

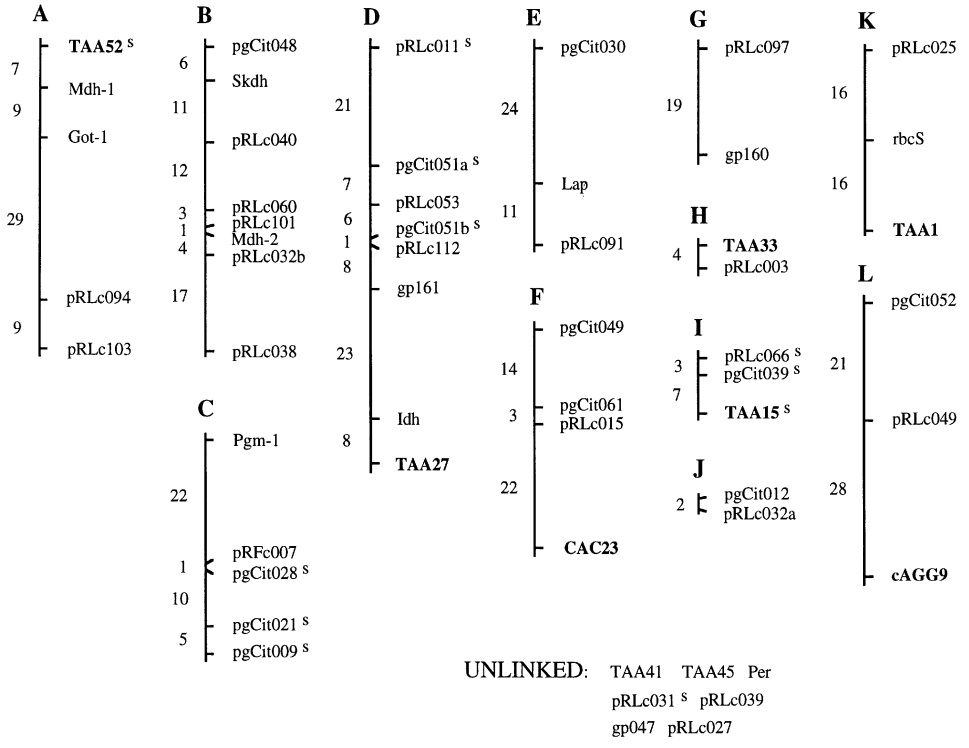

Fig. 2 Linkage map of citrus showing the assignment of seven microsatellites. RFLP markers are identified with '*pRLc*', '*pgCIT*' and '*pg*' depending if they originated from a cDNA library, a *Pst*I *Citrus* genomic library or a *Pst*I *Poncirus* genomic library respectively. Microsatellite markers are in *capital bold type* and the remaining loci are identified by isozyme and protein markers. Those markers showing skewed segregation are denoted using a superscripted 's'

microsatellite markers are assigned to the same linkage group, indicating a widespread distribution within the genome. Two microsatellites, TAA41 and TAA45, segregated independently and remain unlinked to any other marker.

Map coverage and distance calculations

Addition of the microsatellite markers formed two new linkage groups named K and L (Fig. 2). The largest linkage group in the map contains eight markers and covers 74 cM while the smallest contains only two markers and covers 2 cM. JoinMap calculated a map covering a total of 410 cM. Placement of microsatellites at the termini of linkage groups has expanded the map coverage from the previous calculated size of 351 cM; however, direct comparison is difficult as the linkage program MapMaker (Lander et al. 1987) was used to construct the original map (Jarrell et al. 1992). For comparison, JoinMap was used to reconstruct the original map from the segregation data (excluding microsatellite marker information) and a total map length of 279 cM was obtained. This revealed that JoinMap calculates reduced map distances in comparison with MapMaker, a result also reported by Cai et al. (1994). The membership of all linkage groups was the same when calculated with either JoinMap or MapMaker; however, rearrangements to the linear order within two linkage groups was observed. Isozyme marker Mdh-2 changed position within group B and cDNA RFLP marker pRLc053 changed position within group D. In each case the previous location of the marker had been achieved using two-point maximum-likelihood estimates (Jarrell et al. 1992) suggesting that such estimates may not be suitable for accurately determining map location.

Segregation analysis

Due to the very wide nature of the mapping cross, combined with the varied level of polymorphism displayed between microsatellite markers, 1:1, 1:2:1 and 1:1:1:1 segregation ratios were observed (Table 2). Comparison of expected and observed segregation ratios showed two of the nine microsatellite markers to have a significant deviation from the expected ratio ($P > 0.05$). Table 2 also shows that, both the skewed microsatellite markers were of the 1:2:1 segregation type and both were mapped (Table 1 and Fig. 2). Interestingly, one of the two (TAA15) was found to be linked with skewed RFLPs on linkage group I and, in both cases, examination of allele inheritance showed a deficiency of the genotype comprising the homozygote for the *Citrus*-donated alleles. In the case of TAA52 this was accompanied by an excess of *Citrus*/*Poncirus* heterozygote genotypes while for the other (TAA15) it was accompanied by an excess of *Poncirus* homozygotes.

Table 2 Segregation analysis of microsatellite markers

Locus	Expected ratio	Progeny segregation	χ^2
TAA1	1:2:1	11:32:10	2.32
TAA15	1:2:1	23:22:9	9.11*
TAA27	1:1:1:1	17:15:11:9	3.08
TAA33	1:1	25:30	0.45
TAA41	1:1:1:1	9:14:18:14	2.97
TAA45	1:1	33:23	1.79
TAA52	1:2:1	14:32:9	6.32*
CAC23	1:1:1:1	17:15:8:17	3.84
cAGG9	1:2:1	13:34:9	3.14

*$P > 0.05$

Discussion

A total of 14 primer pairs were tested for amplification and product-length polymorphism within a citrus population previously used for linkage mapping. Five primer pairs generated PCR products that were unsuitable for linkage analysis for one of three reasons. Firstly, there was an absence of length polymorphism for two markers (Table 1). A second problem encountered was the amplification of up to six bands per diploid genome at one marker (Table 1). Interestingly, inspection of progeny band patterns suggested amplification of a microsatellite array duplicated within the genome; however, the overlapping allele sizes made treatment as separate markers impossible. A third problem of allele non-amplification was also detected at two markers (Table 1). This has previously been reported in human (Callen et al. 1993; Koorey et al. 1993), deer (Pemberton et al. 1995), bear (Paetkau and Strobeck, 1995) and grapevine genomes (Thomas et al. 1994). The cause of allele non-amplification was shown to be an eight-base pair deletion occurring within the primer sequence for PCR amplification in one instance (Callen et al. 1993), while a single base substitution was sufficient to prevent allele amplification at a separate locus (Koorey et al. 1993). The basis for allele non-amplification at TAA3 and CAC19 was not characterised; however, the presence of these alleles is not surprising considering the very wide intergeneric nature of the breeding cross used for mapping. The presence of null alleles also has implications for marker application within cultivar identification and, if the basis was characterised, may become potentially taxonomically informative.

The first seven trinucleotide microsatellite markers have been added to the *Citrus* genetic map. Their location, in every instance, was found to be on a different linkage group, indicating a widespread distribution throughout the genome. Similar results have been reported in *Arabidopsis* (Bell and Ecker 1994) where the distribution of 30 dinucleotide microsatellites was found to be random. Additionally, a similar result after the map assignment of 40 dinucleotide and trinucleotide microsatellites was obtained within soybean (Akkaya et al. 1995). The only study to-date where a clustering of microsatellites has been detected within a plant genome is for tetranucleotide repeats which were found not to be evenly distributed within the tomato genome (Arens et al. 1995). The present study examined trinucleotide-type repeats and has confirmed the suitability of this marker type for constructing linkage maps within plants due to its broad and random genomic distribution. The assignment of every microsatellite in this study showed placement to the termini of a different linkage group. The genome of *Citrus* has nine haploid chromosomes and has been estimated to cover between 1500–1700 cM (Jarrell et al. 1992). The largest linkage group calculated in this study covered 74 cM indicating that even the largest linkage group is unlikely to completely cover a chromosome. The placement of all microsatellites to the termini of linkage groups is therefore more likely a function of the small number of markers within the map than to be reflective of a non-random distribution of this marker type towards the telomeres of the chromosomes.

Analysis of allele segregation revealed two of the nine microsatellites (22%) displayed a significant deviation from the expected ratio when tested using chi-square (χ^2) goodness-of-fit values (Table 2). A direct comparison can be made with the proportion of distorted segregation occurring at RFLP (9/37, 24%) and isozyme markers (0/8) analysed in the same population (Jarrell et al. 1992). Other studies within citrus have detected higher levels of marker distortion; for example, Durham et al. (1992) found 37% of RFLPs were skewed (21/57) while an even higher proportion was detected while extending the same map with RAPD markers (107/266, 40%, Cai et al. 1994). Although only a limited number of microsatellites have been tested, the proportion of skewed examples appears not to be prohibitively high, especially considering that the map of Cai et al. (1994) was constructed in a backcross population expected to be less sensitive to skewing than the F_2 population used here (Causse et al. 1994). It has previously been observed that markers showing aberrant segregation cluster together on particular regions of the genetic map. Almost half of skewed rice RFLPs (46/96, 48%) were assigned to chromosome 3 (Causse et al. 1994), and within citrus over 40% (31/77) of the mapped RAPDs showing skewed segregation clustered on linkage group I (Cai et al. 1994). In the present study both of the skewed microsatellite markers were mapped and TAA15 was indeed found closely linked to RFLPs showing skewed segregation on group I (Fig. 2). Marker TAA52 mapped to group A and was not linked to other skewed markers (Fig. 2). Examination of the direction of segregation distortion (i.e. between *Citrus* and *Poncirus* parent alleles) showed that in both cases the *Citrus* allele was under represented. This is in agreement with the findings of Jarrell et al. (1992) where the *Citrus* allele was under-represented in eight of nine skewed RFLPs. This

unequal allele segregation toward the *Poncirus* parent appears independent of marker type and also independent on the type of polymorphism being assayed. For plant breeders, the dominance of *Poncirus* should be considered when designing breeding crosses aimed at introgressing specific genes from this breeding parent, and this phenomenon may be a general occurrence in wide citrus crosses.

This study has provided further evidence that microsatellite markers will become an important mapping tool within plants. Specific to citrus, their co-dominant mode of inheritance and amplification within a wide breeding cross should mean that the same markers can be typed and mapped in different breeding crosses. This will allow them to be used as anchor loci to align the RFLP- and RAPD-based linkage maps generated in different mapping crosses from different laboratories. Future application could see them used to align the 11 linkage groups within this map to the nine linkage groups of the RAPD and RFLP map of Cai et al. (1994).

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