Turk J Bot
31 (2007) 79-86

Research Article

Construction of a Genetic Linkage Map of *Citrus* with Random Amplified Polymorphic DNA (RAPD) Markers Using a Progeny Population from a Complex Intergeneric Cross

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Received: 30.09.2005
Accepted: 15.11.2006

Abstract: This study included 30 plants that were randomly selected from a larger progeny population obtained from a complex intergeneric cross of {*C. grandis* (L.) Osb. x [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]}. Genomic DNA was extracted from leaf samples of these plants and analysed for polymorphisms by polymerase chain reaction (PCR), using 10-mer random primers. A total of 111 random amplified polymorphic DNA (RAPD) markers were identified using 38 random primers. A genetic linkage map of the progeny population was constructed with these RAPD markers. The map contains 63 markers distributed into 9 linkage groups, which possibly correspond to the 9 haploid chromosomes of *Citrus*. The total maximum length of the linkage map generated in this study was 314.8 cM, with an average map distance of 5.07 cM between markers.

Key Words: Citrus, DNA markers, RAPD markers, genetic linkage mapping

Rastgele Çoğaltılmış DNA (RAPD) Markörleri ve Cinsler Arası Karmaşık Melez Populasyonu Kullanılarak Turunçgil Genetik Haritasının Oluşturulması

Özet: Cinsler arası kamaşık {*C. grandis* (L.) Osb. x [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]} çaprazlanmasından elde edilen bitki populasyonu içinden 30 ağaç rastgele seçildi. Bu bitkilerden toplanan yaprak örneklerinden genomik DNA ekstraksiyonu yapıldı. Elde edilen DNA'lar ve 10'mer rastgele primerler kullanılarak polymeraz zincir reaksiyonuyla (PCR) polimorfizim analizi yapıldı. Kullanılan otuzsekiz farklı random primerden toplam 111 rastgele çoğaltılmış polimorfik DNA (RAPD) markörü belirlendi. Bu markörlerle seçilen bitki populasyonunun bağlantı haritası oluşturuldu. Bu genetik harita dokuz farklı bağ grubuna dağılmış 63 RAPD markör içermekte olup, bu bağ grupları büyük olasılıkla turunçgillerin dokuz olan haploid kromozom sayısına denk gelmektedir. Bu çalışmada oluşturulan genetik haritanın toplam uzunluğu 314,8 cM ve markörler arasındaki ortalama harita aralığı da 5,07 cM olarak belirlenmiştir.

Anahtar Sözcükler: Turunçgiller, DNA Markörleri, RAPD Markörleri, Genetik Haritalama

Introduction

Genetic linkage mapping is a method in which locations of genes on a chromosome are determined based on recombination frequencies observed in pedigrees or progeny populations (Clark & Wall, 1996). Polymorphism between individuals in a population and detection of these polymorphisms with genetic markers are essential elements of linkage mapping. Construction of a genetic linkage map requires selection of an appropriate population, defining markers or genes, calculation of pair-wise recombination frequencies, establishment of linkage groups and estimation of map distances, and determination of gene or marker order (Staub et al., 1996). Several computer software programs have been developed for the analysis of genetic data for construction of linkage maps, including Linkage

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1 (Suiter et al., 1983), GMendel (Echt et al., 1992), Mapmaker (Lander & Botstein, 1986; Lander et al., 1987), MapManager (Manly & Elliot, 1991), and JoinMap (Stam, 1993; Stam & Van Ooijen, 1995).

Genetic linkage maps were first developed using morphological markers and later, more comprehensive genetic maps were constructed using isozymes. Since the 1980s, a number of DNA-based markers, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), sequence characterised amplified region (SCAR), expressed sequence tagged site (EST), and simple sequence repeat (SSR) markers, have been developed and used for the construction of more complex and informative genetic linkage maps (Mohan et al., 1997). DNA markers are more useful for linkage mapping compared to morphological markers because they use polymorphisms in DNA and are not affected by environmental conditions. Therefore, DNA markers overcome the limitations of morphological markers and isozymes by producing more polymorphisms. Among the DNA-based markers, RFLP and RAPD markers have been most commonly used with plants.

RAPD markers are dominant markers; heterozygotes cannot be distinguished from the homozygote dominant genotype. In addition, RAPDs require PCR optimisation and may be difficult to reproduce in different populations (Kochert, 1994). On the other hand, RAPDs generate more polymorphisms between closely related genotypes, require very small amounts of genomic DNA, and enable screening of large populations of breeding materials because their analysis is quick, not labour-intensive, and can be automated (Deng et al., 1995). Because of these advantages, RAPD markers have been used for linkage mapping, marker-assisted selection, genotype identification, taxonomy, and other genetic studies of cereals, crop plants, and vegetable and fruit crops, including *Citrus* (Mohan et al., 1997).

Citrus is suitable for genetic linkage mapping studies because it is diploid, has only 9 haploid chromosomes, and has a relatively small genome of about 1500-1700 cM (Jarrell et al., 1992). Since the application of mapping studies to *Citrus*, several genetic linkage maps have been constructed using molecular and biochemical markers. Torres et al. (1985) identified the first genetic linkages using isozymes in *Citrus* and *Poncirus*. Later, RFLP and isozyme markers were used to generate the first genetic linkage map of *Citrus*, containing 11 linkage groups (Durham et al., 1992; Liou et al., 1996). Another linkage map in *Citrus* was produced based on the segregation of isozymes and RFLP markers using an intergeneric hybrid of 'Sacaton' citrumelo (*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) and 'Troyer' citrange [*C. sinensis* (L.) Osb. x *P. trifoliata* (L.) Raf.)] (Jarrell et al., 1992). Cai et al. (1994) developed a more complex linkage map of *Citrus* with 9 linkage groups, possibly corresponding to the haploid chromosome number of *Citrus*, with RAPD and RFLP markers using BC_1 progeny from an intergeneric cross of *Citrus grandis* (L.) Osb. x [*Citrus grandis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.]. This map was used for the identification of QTLs associated with Na^+ and Cl^- accumulation-related traits and morphological traits under saline and non-saline conditions (Tozlu et al., 1999a, 1999b).

After the development of microsatellite markers in other crops, Kijas et al. (1995) isolated 2 microsatellite markers from an intergeneric cross between rangpur lime (*Citrus limonia* Osb.) and trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and showed conservation of these markers in the *Citrus* genome. Later, Kijas et al. (1997) added 7 microsatellite markers to the *Citrus* map constructed by Jarrell et al. (1992). To increase the density of the linkage map constructed by Cai et al. (1994), 75 microsatellite markers were added (Sankar et al., 2001). To identify quantitative trait loci (QTL) associated with freeze tolerance, Weber et al. (2003) constructed a map using a *Citrus grandis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf. F_1 pseudo-testcross population. They used different kinds of markers to establish a linkage map for each parent, including RAPD, CAPS, SCAR, and STS markers. In addition to these maps, a few other maps have been constructed for *Citrus* using different species, including *C. grandis* (L.) Osb. (Luro et al., 1996), *C. aurantium* L. and *C. latipies* (Simone et al. 1998), *C. sunki* Hort. ex Tan. and *P. trifoliata* (L.) Raf. (Cristofani et al. 1999), and *C. volkameriana* Ten. and *P. trifoliata* (L.) Raf. (Garcia et al. 1999). To compare the *Citrus* and *Poncirus* genomes, Ruiz and Asins (2003) established 5 genetic linkage maps of the parents of 3 progenies: *C. aurantium* (L.) (A) x *P. trifoliata* (L.) Raf. var. *Flyin Dragon* (Pa), *C. volkameriana* Ten. (V) *P. trifoliata* (L.) Raf. var. *Rubidoux* (Pv), and a self-pollination of *P. trifoliata* (L.) Raf. var. *Flying Dragon* (Pp), using ESTs, SSRs, and inter-retrotransposon amplified polymorphism (IRAP) markers. Among these 5 linkage maps, 2 constructed using a *Citrus aurantium* (L.)

(A) x *Poncirus trifoliata* (L.) Raf. var. *Flying Dragon* (Pa) population were updated by increasing family size and markers used for the establishment of the linkage group to identify QTLs involved in CTV accumulation (Asins et al., 2004). The same linkage map was used for QTL analysis of citrus leafminer (CLM) resistance and 2 other traits might be related to it: deciduousness and leaf area of the tree (Bernet et al., 2005).

Although to date several genetic linkage maps have been established using different markers in *Citrus*, these maps were generally constructed using progenies from 2 different *Citrus* species or *Citrus* relatives. In this study, a genetic linkage map of *Citrus* was constructed with RAPD markers, using progeny from a complex intergeneric cross {*C. grandis* (L.) Osb. *x* [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]}. This is the first genetic linkage map of a complex intergeneric cross of these commercially important *Citrus* species.

Materials and Methods

Plant Materials: A progeny population of 30 plants obtained from the {*C. grandis* (L.) Osb. *x* [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]} intergeneric cross was used in this study (Figure 1). The plants were maintained in the experimental orchard of the Horticultural Sciences Department at the University of Florida, Gainesville, Florida, USA.

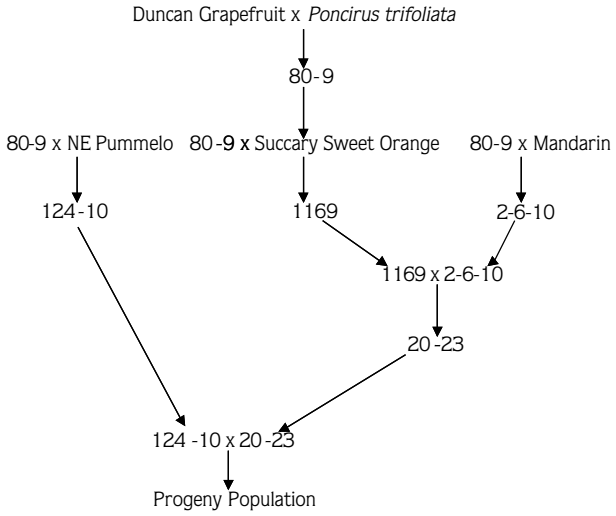

Figure 1. Genetic background of the progeny population.

DNA Extraction: DNA was extracted from 100 mg of leaf tissue in 1.5 ml Eppendorf tubes using the DNA extraction method described by Edwards et al. (1991)

Oligonucleotide Primers: A total of 111 random 10-mer oligonucleotide primers from A, B, C, E, G, O, Q, and R kits (Operon Technologies Inc.) were used to amplify *Citrus* genomic DNA. These primers were chosen mostly based on the rate of polymorphism generated in previous studies involving *Citrus* genome mapping (Cai et al., 1994; Weber et al., 2003).

Polymerase Chain Reaction (PCR): PCR amplification was conducted with 50 µl of reaction mixture containing PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 1% Triton X-100), 2 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM primer, 2 units of *Taq* polymerase (Promega), and 30 ng of genomic DNA. The reaction mixture was overlaid with 2 drops of mineral oil. The amplification reaction was carried out in a Thermocycler PTC-100 with 60 wells (MJ Research, Inc.). The thermocycler was programmed for 1 cycle of 1-min initial denaturation at 93 °C and for 42 cycles of 1-min denaturation at 92 °C, 1-min primer annealing at 35 ºC, a 2-min primer extension at 72 ºC, followed by 1 cycle of final primer extension at 72 ºC for 10 min.

Gel Electrophoresis and Staining: PCR products were separated in 1.5% agarose gel by electrophoresis in TAE buffer (0.5 M Tris-base, 0.5 M EDTA (pH 8.0), and 12.6% glacial acetic acid) with a constant power of 60 V for about 2 h. The products were stained with ethidium bromide and then visualised and photographed under UV light using the IS-1000 digital gel imaging system (Alpha Innotech Corp.).

Analysis of Polymorphism: To detect polymorphisms, all primers were initially screened using parents and 5 of the progeny. The primers producing polymorphic bands were then used to screen a progeny population of 30 plants. The stable amplification products were scored as + for the presence of and – for the absence of a specific band. The specific bands showing polymorphism were named with the primer letter and number followed by the approximate size of the amplified fragments.

Construction of Genetic Linkage Map: Markers showing a 1:1 segregation ratio were scored for each progeny as ab for the presence of and aa for the absence of the bands. The markers showing a 3:1 segregation ratio were scored as b- for the presence of and aa for the absence of the bands for each progeny. The linkage analysis was performed with JoinMap version 2.0 (Stam and Van Ooijen, 1995). A logarithm of the odds ratio (LOD) score of 3.0 was established for linkage and Kosambi's mapping function was used to determine map distances in centimorgans (cM), based on recombination frequencies. After construction of a core map with a LOD level > 3, each linkage group was reconstructed with lower LOD scores (between 2 and 2.9) to distribute some of the unlinked markers to the core map.

Results and Discussion

Generation and Analysis of RAPD Markers: Among the 111 random primers used for initial screening of the parents and 5 progeny, 73 primers either failed to amplify any DNA bands, or produced no polymorphism or inconsistent polymorphism. These primers were not used for further analysis. The rest of the random primers (38, which is about 34% of all random primers tested) produced consistent and obvious polymorphic DNA fragments, and were subsequently used for screening the genomic DNA of the 30 plants from the progeny population for segregation and genetic linkage analysis. The numbers of amplified DNA fragments, and polymorphic and scorable bands obtained from individual primers are shown in Table 1. The percentage of random primers that produced polymorphisms in the progeny population used in this study was considerably lower than in a previous study with BC_1 progeny from *Citrus grandis* x [*Citrus grandis* x *Poncirus trifoliata*] in which 49% of the primers showed polymorphism (Cai et al., 1994). Although some of the primers used in this study were the same as the primers used in a previous study by Weber et al. (2003), we observed fewer polymorphisms per primer than they did. These variations in the number of polymorphisms of this study and these 2 previous studies might be due to differences in the characteristics of the mapping populations. Amplification of genomic DNA from the progeny population with 38 random primers produced a total of 329 bands and a single primer amplified 2-16 DNA bands ranging from 200 bp to 3kb. Out of 329 amplified products, 111 showed reproducible polymorphisms, and were scored and used for linkage analysis (Table 1). An agarose gel profile of amplified products is shown in Figure 2.

Table 1. List of random primers used and the number of amplified, polymorphic DNA bands, and mapped loci produced by individual primers from the progeny population.

Primer	Sequence	Number of bands		
		Amplified	Polymorphic and Scorable	Mapped
OPA02	TGCCGAGCTG	12	2	2
OPA04	AATCGGGCTG	5	1	1
OPA18	AGGTGACCGT	8	4	3
OPB01	GTTTCGCTCC	6	4	3
OPB04	GGACTGGAGT	7	3	2
OPB05	TGCGCCCTTC	10	3	3
OPB10	CTGCTGGGAC	7	2	2
OPB17	AGGGAACGAG	13	4	1
OPB18	CCACAGCAGT	9	1	1
OPC02	GTGAGGCGTC	14	6	4
OPC04	CCGCATCTAC	14	4	2
OPC05	GATGACCGCC	16	3	1
OPC08	TGGACCGGTG	9	3	1
OPC11	AAAGCTGCGG	9	3	2
OPC12	TGTCATCCCC	5	1	
OPC13	AAGCCTCGTC	6	4	3
OPC19	GTTGCCAGCC	5	2	2
OPE01	CCCAAGGTCC	10	1	
OPE02	GGTGCGGGAA	9	1	1
OPE04	GTGACATGCC	10	6	1
OPE06	AAGACCCCTC	9	1	
OPE07	AGATGCAGCC	13	7	4
OPE11	GAGTCTCAGG	9	2	2
OPE14	TGCGGCTGAG	12	5	3
OPE15	ACGCACAACC	13	5	3
OPE16	GGTGACTGTG	4	1	1
OPE17	CTACTGCCGT	2	2	
OPE18	GGACTGCAGA	9	5	1
OPE20	AACGGTGACC	10	2	
OPG05	CTGAGACGGA	4	1	1
OPG11	TGCCCGTCGT	6	2	
OPO13	GTCAGAGTCC	3	1	
OPQ01	GGGACGATGG	12	8	5
OPQ18	AGGCTGGGTG	10	6	2
OPR02	CACAGCTGCC	10	2	1
OPR08	CCCGTTGCCT	4	1	1
OPR09	TGAGCACGAG	6	2	1
OPR12	ACAGGTGCGT	9	3	3

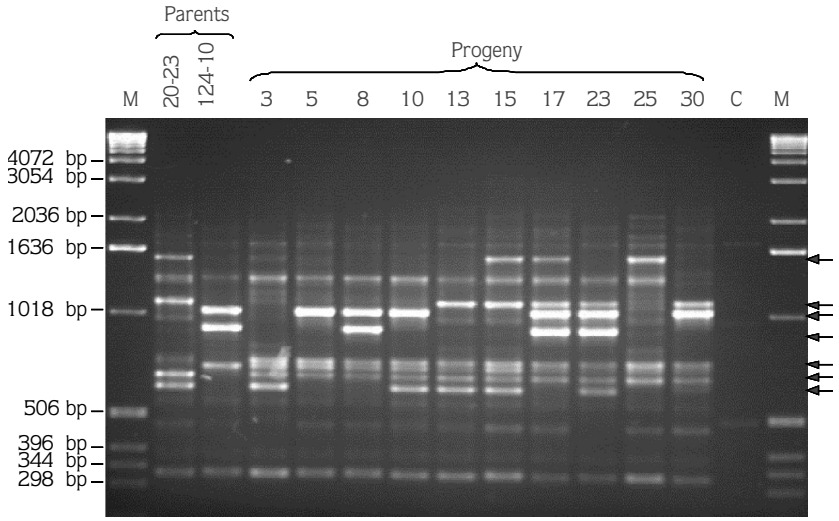

Figure 2. Ethidium bromide staining of DNA fragments amplified with OPE07 random primer by RAPD PCR and separated on 1.5% agarose gel by electrophoresis. M indicates 1 kb molecular weight marker, C indicates water control with no DNA, and arrows indicate polymorphic bands.

In this study, reproducibility of DNA bands was tested for all the primers, first in the parents and 5 progeny. Then, only primers producing consistent polymorphism were used in the mapping population of the 30 progeny. A number of polymorphic bands were not scored because they were too faint and inconsistent, and sometimes amplified differently in repeated experiments; therefore, we confirmed the reproducibility of RAPD markers and used only reproducible and reliable ones for map construction.

Segregation Analysis and Linkage Mapping: Using 38 random primers, 111 polymorphic loci were identified. Among these polymorphic loci, 59 showed an aa x Aa genotype in which a band was present in 20-23 and absent in 124-10, 38 were identified as Aa x aa where a band was absent in 20-23 and present in 124-10, and 14 were classified as Aa x Aa in which a band was present in both parents (Table 2). Alleles at the 97 loci showing either aa x Aa or Aa x aa were expected to segregate in a 1:1 ratio and they were tested for goodness of fit using chi-square analysis. Since loci with an Aa x Aa genotype were expected to show 3:1 segregation, they were tested for goodness of fit to that ratio. Chi-square analysis demonstrated that all of the loci that were expected to segregate 3:1 and 83 of the 97 loci expected to segregate 1:1 fit the expected segregation ratio. Distorted segregations ($P < 0.05$) were observed for 14 markers (about 13%), with 6 of them skewed towards 20-23 and 8 of them skewed towards 124-10. The percentage of distorted segregation observed in this study was significantly lower than that found in previous studies in *Citrus*, where RFLP markers showed 37% segregation distortion (Durham et al., 1992), RAPD markers exhibited 40% (Cai et al., 1994), a combination of RFLP and isozymes demonstrated 20% (Jarrell et al., 1992), and microsatellite markers displayed 22% segregation distortion (Kijas et al., 1997). When the distribution of markers were evaluated, more than 50% (60) of the marker loci were associated with one of the linkage groups, including 29, 26, and 8 markers, with aa x Aa, Aa x aa, and Aa x Aa genotypes, respectively. Two linkage groups included markers with 3 different genotypes; however, the remaining 7 linkage groups contained either aa x Aa or Aa x aa genotypes.

Segregation of RAPD markers showed that 59 of 111 RAPD marker loci were heterozygous in parent 124-10 and only 38 of 111 RAPD marker loci were heterozygous in parent 20-23. These results may be explained by the genetic background of the parents. Parent 124-10 was obtained from the complex cross of {[(*C. paradisi* x *P. trifoliata*) x *C. reticulata*] x [(*C. paradisi* x *Poncirus trifoliata*) x *C. sinensis*]}, but the other parent, 20-23,

Table 2. Summary of marker genotypes and their distribution to the linkage groups.

Linkage Groups	Number of Markers	Genotypes of the Markers		
		aa x Aa	Aa x aa	Aa x Aa
I	6	0	6	0
II	12	8	2	2
III	16	6	4	6
IV	5	5	0	0
V	6	6	0	0
VI	6	0	6	0
VII	4	4	0	0
VIII	5	0	5	0
IX	3	0	3	0
Unlinked	51	31	13	7
Total	111	59	38	14

was obtained from the relatively simple cross of {*C. grandis x* [*C. paradisi* x *Poncirus trifoliata*]}. Therefore, it was expected that 124-10 would be more heterozygous than 20-23, since it was generated from 3 citrus species and 1 relative.

Segregation data from the 111 RAPD markers were analysed for linkage mapping using JoinMap version 2.0 (Stam and Van Ooijen, 1995). Three-point analysis showed that 63 of the 111 markers were linked with an LOD level > 3 and fell into 9 linkage groups containing 3-16 markers (Figure 3). Among the remaining 49 unlinked markers, there were 7 linked pairs, including E04093/E15043, A18050/B04081, C19115/B10037, E18099/C12039, E20083/Q01061, Q18118/O13052, and C0496/B17069, and 1 group with 3 markers (E07103 /E18066 /B04075) that did not show linkage with the LOD level of > 3. Among the 9 linkage groups, only II and III contained RAPD marker loci with all genotypes. The other groups included markers only of genotype Aa x aa (I, VI, VIII, and IX) or aa x Aa (IV, V, and VII) (Table 2). The markers with genotype Aa x Aa showed linkage with Aa x aa and aa x Aa. Since markers with Aa x aa and aa x Aa genotypes came from one of the parents, the linkage between these markers can only be detected if one of them linked to a marker with Aa x Aa genotype, which was present in both parents.

Conclusions

A genetic linkage map was constructed with RAPD markers using a progeny population from a {*C. grandis* (L.) Osb. *x* [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]} intergeneric cross. The map contains 63 markers distributed into 9 linkage groups possibly corresponding to the 9 haploid chromosomes of *Citrus* (Figure 3). Total maximum length of the linkage map generated in this study was 314.8 cM, with an average map distance of 5.07 cM between markers. The length of individual linkage groups ranged from 10.1 to 63.5 cM. The largest distance between markers was 27.2 cM and this was only observed in one linkage group; most of the markers had interval distances of 5.07 cM. The linkage groups I, II, and III contained more than 50% of the total markers. The size and the distance between the markers in this study were smaller than those generated in previous mapping studies of *Citrus* (Durham et al., 1992; Cai et al., 1994). The genome of *Citrus* has been estimated to span 1500 to 1700 cM (Liou et al., 1990). The map generated in this study covers about 18% to 21% of the genome and it can be extended by the addition of more markers.

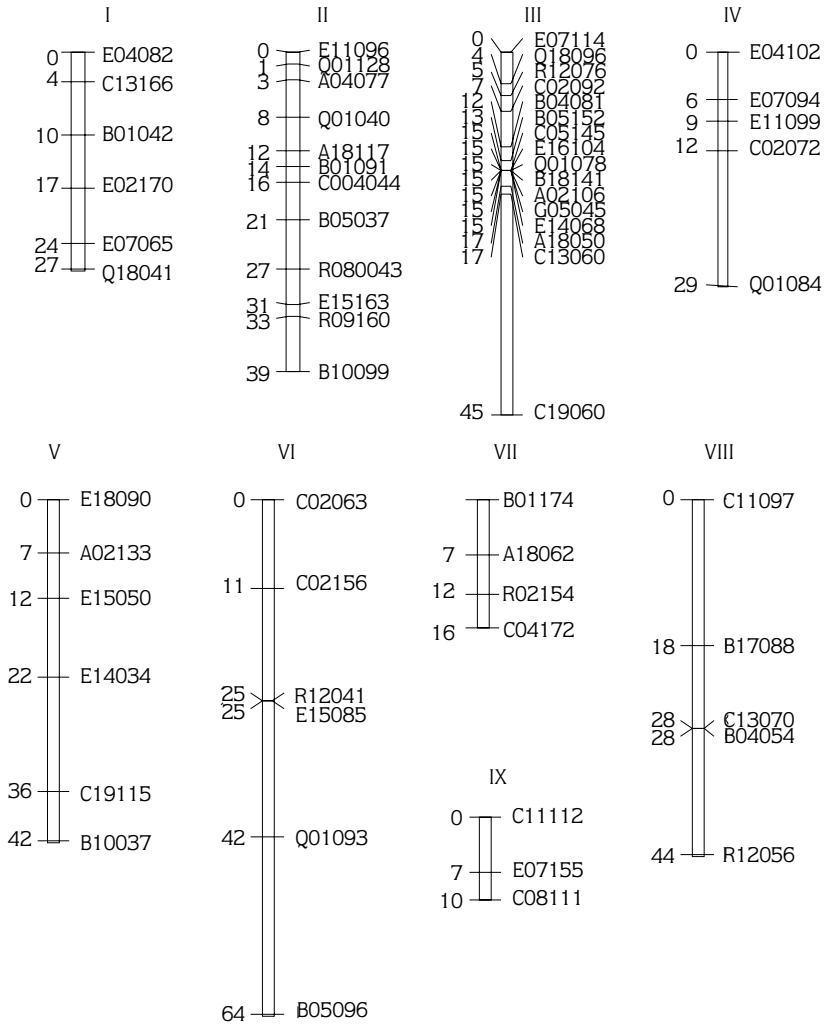

Figure 3. Linkage map of *Citrus* constructed with RAPD markers using {*C. grandis* (L.) Osb. *x* [*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.]} x {[(*C. paradisi* Macf. x *P. trifoliata* (L.) Raf.) x *C. reticulata* Blanco] x [(*C. paradisi* Macf. x *Poncirus trifoliata* (L.) Raf.) x *C. sinensis* (L.) Osb.]}.

This study demonstrated that RAPD markers are useful for the construction of a linkage map because a sufficient number of markers can be generated and used for construction in a relatively short period of time. The stability and reproducibility of RAPD markers was also confirmed since more than 50% of the polymorphisms observed showed linkage and mapped to a specific linkage group in this study.

The population used in this study was originally obtained for providing cold-hardy citrus cultivars. Since this population contains different citrus cultivars, it can be used for studying morphological traits in *Citrus* as well as other commercially important traits, such as biotic and abiotic stress tolerance, and fruit quality and productivity. Thus, the initial linkage map generated in this study can be extended by the addition of more molecular markers, such as SSR, AFLP, RFLP, and SCARs, and can be used for QTL analysis of morphological and other traits in *Citrus*. This map may also be integrated with other linkage maps of *Citrus* containing different markers to generate a more comprehensive linkage map. It may also be used for the characterisation of economically important traits and marker-assisted selection for breeding studies.

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