Theor Appl Genet (1992) 84:49–56

THEORETICAL AND APPLIED GENETICS

A genetic map of citrus based on the segregation of isozymes and RFLPs in an intergeneric cross

D. C. Jarrell*, M. L. Roose, S. N. Traugh, and R. S. Kupper

Department of Botany and Plant Sciences, University of California, Riverside, Riverside, CA 92521, USA

Received September 24, 1991; Accepted October 9, 1991
Communicated by F. Salamini

Summary. Isozymes and restriction fragment length polymorphisms were used as markers in the construction of a genetic map of the citrus nuclear genome. The map was based on the segregation of 8 isozyme, 1 protein, and 37 RFLP loci in 60 progeny of a cross of two intergeneric hybrids, 'Sacaton' citrumelo (*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.) and 'Troyer' citrange (*C. sinensis* (L.) Osbeck × *P. trifoliata*), often used as rootstocks. The map contains 38 of 46 studied loci distributed on ten linkage groups. A genome size of 1,700 cM was estimated from partial linkage data. Approximately 35% of the genome should be within 10 cM and 58% within 20 cM of the mapped markers. Eight loci in three linkage groups and 1 unlinked locus deviated significantly from Mendelian segregation.

Key words: Linkage mapping – Citrus – Poncirus – Molecular markers

Introduction

Breeding of citrus and many other fruit trees is a long, tedious process complicated by long periods of juvenility, insufficient space and, in citrus, a high degree of apomixis. The efficiency of tree breeding programs could be improved if molecular marker-based maps were available. Loci affecting traits such as fruit quality, disease resistance and environmental tolerances can be added to such maps. If tight linkages are found between markers and such loci, selection for the markers should allow breeders to develop populations with a high frequency of the linked genes. Breeders could then apply more intense selection for other traits such as yield and quality.

Restriction fragment length polymorphisms (RFLPs) and isozymes facilitate detailed mapping because they are highly polymorphic in many species, are codominant, and do not have deleterious phenotypic effects, epistasis, or pleiotropy. RFLPs result from differences at the DNA level, including base changes in restriction sites and insertions or deletions. In many species, a large number of markers can be identified in a single cross (Tanksley 1983; Soller and Beckman 1983). Molecular marker-based maps of tomato (Tanksley and Rick 1980; Helentjaris et al. 1986; Bernatzky and Tanksley 1986) pepper (Tanksley et al. 1988), potato (Bonierbale et al. 1988), and lentil (Havey and Muehlbauer 1989) were developed using interspecific crosses due to a low level of intraspecific polymorphism. While it is often desirable to work within more closely related germ plasm (Helentjaris 1987; McCough et al. 1988; Gebhardt et al. 1989), these and other maps have enabled marker-based introgression of desired genes from wild germ plasm (Tanksley and Rick 1980; Young and Tanksley 1989; Sarfatti et al. 1989), comparison of genome organization and evolution (Tanksley et al. 1988); Bonierbale et al. 1988), and analysis of quantitative trait loci (QTL) (Tanksley et al. 1982; Nienhuis et al. 1987; Tanksley and Hewitt 1988; Paterson et al. 1988; Martin et al. 1989; Keim et al. 1990).

Citrus and other long-generation species have lagged behind annuals in genetic studies and mapping. Several problems hinder genetic mapping in citrus. Inbreeding is nearly impossible because of long generation times and the predominance of inbreeding depression in narrow crosses (Cameron and Frost 1968). Consequently, mapping must be conducted in populations derived from heterozygous parents, and phase relationships are not al-

* Correspondence to: D. C. Jarrell

ways known. Finally, in citrus, progeny from sexual reproduction often have to be distinguished from a large number of apomictic progeny. Isozymes and, in certain cases, morphological markers allow apomictic seedlings to be identified (Soost et al. 1980; Torres et al. 1982; Moore and Castle 1988; Xiang and Roose 1988).

Some characteristics of citrus facilitate the construction of genetic maps. Interspecific and even intergeneric hybridization is easy and commonly used by breeders. Such hybrids are highly heterozygous, as has been demonstrated with isozymes (Torres 1983; Torres et al. 1985) and RFLPs (Roose 1988). The citrus relative *Poncirus trifoliata* (L.) Raf. or trifoliate orange is a morphologically distinct, inedible species which, like most other genera in the Citrinae, hybridizes well with various *Citrus* (Swingle and Reece 1967; Soost and Cameron 1975). *Citrus* × *Poncirus* hybrids are often fertile, and several are used as rootstocks due to their inheritance of various *Poncirus*-derived traits such as cold hardiness and resistance to tristeza virus, *Phytophthora* root rots, and the citrus nematode (*Tylenchulus semipenetrans* Cobb). In this paper, we report construction of a genetic map from a *Citrus* × *Poncirus* F_2 population.

Materials and methods

Citrus strains and cross

Two vegetatively propagated trees of 'Sacaton' citrumelo (*C. paradisi* Macf. × *P. trifoliata*) were hybridized with pollen from 'Troyer' citrange (*C. sinensis* (L.) Osbeck × *P. trifoliata*). These parental cultivars are used as rootstocks for citrus. Seedlings derived from hybridization were identified using morphology and 7 isozyme loci that were heterozygous in 'Sacaton'. Of these, 60 were chosen for the linkage analysis, the only selection being against extremely weak seedlings that produced too few leaves for analysis (about 10% of the total progeny). Cultivars selected to represent grandparental lines were *P. trifoliata* 'Pomeroy', *C. paradisi* 'Duncan' and *C. sinensis* 'Olinda Valencia'.

Isozyme and protein analysis

Isozymes were analyzed by starch gel electrophoresis according to Xiang and Roose (1988). Segregation of a gene encoding the small subunit of ribulose 1,5-bis-phosphate carboxylase (rbcS) was analyzed on 8–20% polyacrylamide gradient gels using the TRIS-glycine buffer system (Laemmli 1970). Proteins were extracted by homogenizing 0.2 g of leaf tissue in 0.80 ml 0.05 *M* sodium phosphate buffer, pH 7.0. Cell debris was pelleted at 12 000 *g*, the supernatent removed, centrifuged again, and a equal volume of 2 × SDS loading buffer was added. Extracts were placed in a boiling water bath for 10 min before loading onto gels. Gels were stained with Coomassie Blue. The rbcS subunit was identified as an intensely staining 15-kDa polypeptide which purified as part of a 550-kDa protein that also included a 55-kDa subunit. An RFLP detected with a pea rbcS cDNA clone cosegregated with this polypeptide.

DNA isolation

Two methods were employed in isolating genomic citrus DNA. The first was a modification of a procedure involving a CsCl gradient (Rawson et al. 1982). Approximately 5 grams of nearly mature leaf tissue was collected and held on ice. The tissue was ground to a fine powder using liquid nitrogen in a mortar and pestle, and then extraction buffer was added. Following initial centrifugation and cell lysis, a single CsCl centrifugation was performed at 157 000 *g* in a VTi 65.2 rotor (Beckman). Ethidium bromide was removed by several extractions with NaCl-saturated isopropyl alcohol, and the sample was dialyzed against TE (10 m*M* TRIS, 1 m*M* Na_2-EDTA), pH 8.0 overnight. The DNA was precipitated in 0.1 *M* NaCl and 2 vol ethanol overnight at −20 °C or for 30–60 min at −80 °C. The precipitated DNA was resuspended in 500 µl sterile, double-distilled (dd) H_2O. DNA concentration was measured in a TKO 100 DNA fluorometer (Hoefer Scientific) and adjusted by evaporation or dilution with sterile ddH_2O to approximately 60 µg/ml and, later during the study, to 80 µg/ml. This procedure yielded 20 µg DNA/g fresh weight (fw) tissue.

A second method was sought to produce DNA of sufficient purity in a shorter time. One method which proved quite satisfactory was a slight modification of that used to isolate DNA from filamentous fungi (R. Rodriguez, personal communication). Approximately 0.5 g of young leaf tissue was collected and held on ice for immediate use or stored overnight at −80 °C. The tissue was ground to a fine powder in liquid nitrogen, transferred to a 15-ml centrifuge tube, and 5 ml extraction buffer [150 m*M* Na_2-EDTA, 50 m*M* TRIS pH 8.0, 2.0% sarkosyl, 2 mg/ml type III papaya protease (Sigma)] was added. The slurry was then vortexed for 5 s and incubated at 65 °C for 40–50 min. Cell debris was removed by centrifugation for 30 min at 12 000 *g*. The supernatant was transferred to a new tube, and the DNA precipitated by gentle mixing in 0.7 vol PEG/NaCl solution (20% PEG MW 8000, 2.5 *M* NaCl) and placing on ice for 15 min. The DNA was pelleted at 3000 *g* and resuspended in 2.0 ml TE. After the DNA had dissolved, it was precipitated by gently mixing with 0.5 vol 7.5 *M* ammonium acetate and 0.6 vol isopropyl alcohol and placement on ice for 15 min. The DNA was pelleted and resuspended as before. These two precipitations and resuspensions were repeated. Protein, RNA, and other contaminants were precipitated by adding 0.5 vol 7.5 *M* ammonium acetate, mixing, placing on ice for 30 min, and pelleting at 12 000 *g* for 30 min. The supernatant was transferred to a new tube, 0.6 vol isopropyl alcohol was added, and the DNA precipitated, pelleted as before, and resuspended in 0.5 ml TE.

At this point the original procedure ended, but we found it necessary to add an organic extraction step to isolate citrus genomic DNA readily digested by restriction endonucleases. One volume phenol and 1 vol chloroform : isoamyl alcohol (24 : 1) were added, gently mixed for 5 min at room temperature, and centrifuged at 3000 *g* for 5 min. The aqueous layer was transferred to a new tube, and the DNA was precipitated with ice-cold ethanol (95%) on ice for 15 min and pelleted as before. The pellet was allowed to dry partially and then resuspended in 500 µl sterile ddH_2O. DNA concentration was determined as before, and samples were adjusted to 80 µg/ml. The average yield from this procedure was 200 µg DNA/g fw tissue.

Origin of probes

Probes for RFLP analysis were of three origins. Most probes were inserts from a cDNA library prepared as follows. Total RNA was isolated from the leaves of rough lemon (*C. jambhiri*) using a slight modification of the methods of Silflow et al. (1979) and Murray et al. (1983). Polyadenylated RNA was selected on oligo(dT)-cellulose columns (Maniatis et al. 1982). First strand cDNA was synthesized with AMV reverse transcriptase according to supplier's instruction (BioRad). Second strands were synthesized, and the cDNA was C-tailed (Gubler and Hoffman

1983). cDNAs were cloned into the *Pst*I site of pUC9 (Pharmacia) and transformed into the JM83 *E. coli* strain (Hanahan 1983). Individual clones are designated pRLc, and inserts ranged in size from 0.4 to 1.5 kb.

A genomic library was constructed from the DNA of trifoliate orange cv 'Pomeroy'. Genomic DNA was isolated by the procedure of Rawson et al. (1982) and digested with *Pst*I. Fragments were separated on an agarose gel, and those ranging in size from 0.5 to 1.5 kb were excised and cloned into the *Pst*I site of the phagemid pBluescript II SK m13(+) (Stratagene). XL-1 Blue was transformed according to supplier instructions (Stratagene). No single-copy screen was performed. Selected clones from this library are designated gp.

Cloned inserts from a third library were received from Dr. R. Durham and Dr. G. Moore (University of Florida). The 15 genomic clones (1.1–4.5 kb) of 'Temple' tangor (an orange-mandarin hybrid) were cloned into the *Pst*I site of pTZ18R, and recombinant plasmids were transformed into XL-1 Blue (Stratagene). These clones are designated pgCit.

Restriction digests, electrophoresis, and blotting

Citrus DNA samples were individually digested with the restriction endonucleases *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I, according to manufacturers' (Pharmacia, Boehringer-Mannheim, Stratagene) recommendations except that 10 units of restriction endonuclease per µg DNA and 3 m*M* spermidine were added. Digested DNA samples (2.0 µg/lane) were electrophoresed in 1.0 × TBE 1.0% agarose gels (15 × 18 cm with two 20-well combs/gel) at 50 V for 30 min and 90–95 V for 2.5–3.5 h. The DNA was denatured (1.5 *M* NaCl, 0.5 *M* NaOH, 30 min), neutralized (1.5 *M* NaCl, 0.5 *M* TRIS, pH 7.4, 30 min), and transferred to membranes for 24 h (Southern 1975) using 20 × SSC.

Probe hybridization

Two nylon membranes were used in this study: the positively charged TM-NYX4 (Hoefer Scientific) and the uncharged Magnagraph (MSI) membranes. The TM-NYX4 blots were rinsed in 6 × SSPE for 10–20 min, air dried, and baked under vacuum at 80 °C for 2 h. Prior to probe hybridization, the blots were hydrated for 10 min in 6 × SSPE and prehybridized in 1.0 *M* NaCl, 1% ultrapure SDS at 65 °C for 1–3 h. This was replaced by 25 ml of hybridization solution [0.1 g/ml ultrapure dextran sulphate, 1.0 *M* NaCl, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA (Reed and Mann 1985)] for two 15 × 18-cm blots, and the probe was added. Membranes were hybridized at 65 °C for 24 h. The blots were washed in 1 × SSPE/1% SDS at room temperature for 20 min and again at 65 °C for 20 min. They were then washed in 0.5 × SSPE/1% SDS at 65 °C. Blots were autoradiographed on X-OMAT AR film at −80 °C for 24–48 h. Blots were stripped with 0.01 × SSPE/0.1% SDS at 65 °C for 30–60 min and could be reprobed at least seven times.

In an attempt to reduce background problems encountered with the TM-NYX4 membrane, the DNA was transferred to Magnagraph membranes and UV crosslinked (Church and Gilbert 1984). Blots were washed once for 10 min in 0.5% fraction V-grade BSA/1 m*M* Na_2EDTA/40 m*M* $NaHPO_4$, pH 7.2/5% SDS at 65 °C. This was followed by two to four 5 min washes (1 m*M* Na_2EDTA/40 m*M* $NaHPO_4$, pH 7.2/1% SDS) at 65 °C until the blot gave peak signals of 800–1500 cpm as described above. Blots were autoradiographed and could be stripped and reprobed at least nine times.

Insert isolation and random-primer labelling

To isolate DNA inserts for labelling, bacteria were grown overnight in LB/Amp liquid culture, and plasmids were purified by the boiling method (Maniatis et al. 1982). The samples were digested with *Pst*I according to manufacturer's instructions and treated with DNAse-free RNAse simultaneously. If the DNA was not digestable, it was further purified by the ethanol wash steps of the alkaline lysis procedure (Maniatis et al. 1982). Insert isolation and random primer labelling were slightly modified from the method of Feinberg and Vogelstein (1984). The labelling reaction was carried out at 37 °C for 1 h using 10 ng DNA/50 µl reaction vol. Unincorporated nucleotides and agarose were removed from the labelled probe by chromatography on NACS Prepac cartridges (BRL) according to manufacturers' instructions. Average specific activity of labelled probes was 1.5×10^9 cpm/µg.

Identification of RFLP loci

All pRLc clones were previously hybridized to blots containing digests of a variety of citrus accessions, including the parent cv 'Troyer' and cultivars representing the grandparents of the progeny used in this study (Roose and Traugh, unpublished results). Informative probe/enzyme combinations could usually be determined from these autoradiographs. In cases where confusion existed and with the remaining clones, screening blots were prepared. DNA of the two parental and three grandparental clones was digested with three different enzymes (usually *Eco*RI, *Eco*RV, and *Hin*dIII). Informative probe/enzyme combinations could then be chosen from those that displayed a pattern of inheritance indicative of heterozygosity in one or both parents. RFLP loci were designated by the code of the probe used to detect them. When more than one locus was detected with a particular probe, a lowercase letter was added to identify each locus. RFLP allele sizes are listed in Appendix A of Jarrell (1991).

Analysis of segregation and linkage

Loci were tested for single-locus Mendelian segregation using chi-square (χ^2) goodness-of-fit values. Linkage between loci was tested by χ^2 or, if either locus showed skewed segregation, by contingency χ^2 for significant departure from independent assortment. Two-point maximum likelihood estimates of recombination and standard errors (Allard 1956) were calculated using a BASIC program, X2&MXLIN (Roose, unpublished results). Estimates of recombination were converted to map distances by the Kosambi function (Kosambi 1944). Also, two-point, three-point, and n-point linkage were analyzed on a DECStation 3100 workstation using the F_2 mode of MAPMAKER (Lander et al. 1987; Lander and Green 1987). Loci segregating 1:1:1:1 were converted to 1:2:1 segregations by combining appropriate heterozygous classes. All alleles originating from *P. trifoliata* were designated A, and those from *C. paradisi* and *C. sinensis*, B. A LOD score of 3.0 was used to detect linkage and determine the most likely gene orders. Some loci were heterozygous in only one parent and segregated in a 1:1 ratio. These loci could not be analyzed in the F_2 mode of MAPMAKER and were added to the map manually using map distances calculated from two-point maximum likelihood estimates.

Estimation of map length

Map length was estimated from partial linkage data according to Hulbert et al. (1988). This method requires knowledge of the number of locus pairs linked at predetermined LOD scores. Since only MAPMAKER provided these numbers, this analysis was limited to the 39 loci analyzed with this program.

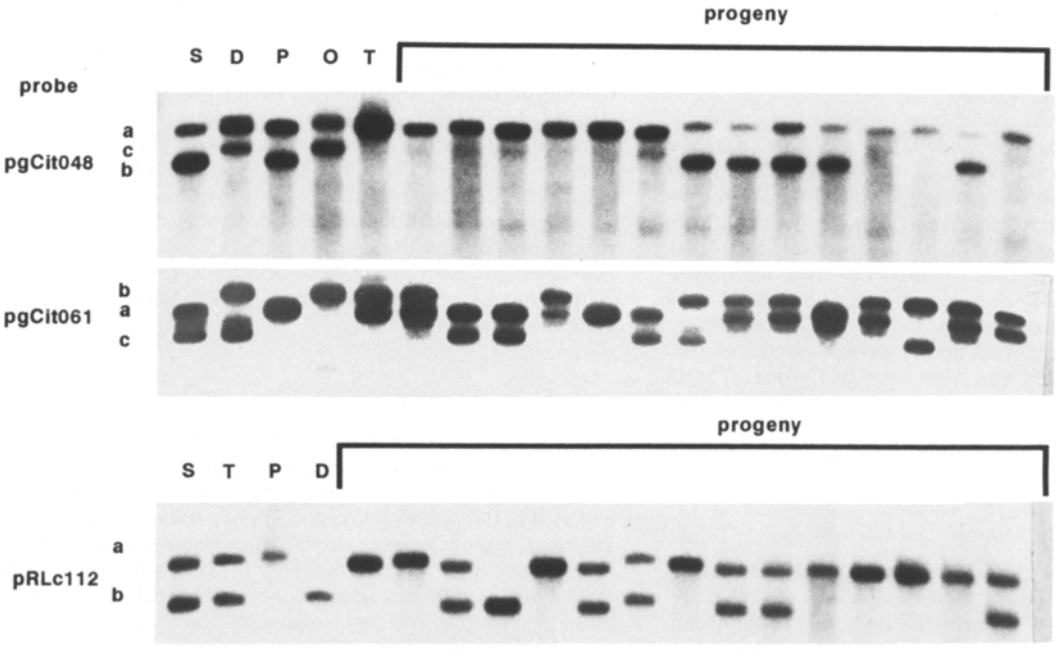

Fig. 1. Autoradiographs of Southern blots of parental and grandparental clones and segregating progeny of the cross of 'Sacaton' citrumelo and 'Troyer' citrange hybridized with genomic clone pgCit048, genomic clone pcCit061, and cDNA clone pRLc112. Aliquots of 1.5–2.0 µg DNA were digested with *Eco*RI (pgCit048, pRLc112) or *Hin*dIII (pgCit061). Parental and grandparental clones are designated as: *S* 'Sacaton' citrumelo, *T* 'Troyer' citrange, *D* 'Duncan' grapefruit, *P* 'Pomeroy' trifoliate orange, *O* 'Olinda Valencia' sweet orange. *Lanes 6–19* (pgCit048 and pgCit061) and *5–19* (pRLc112) are digested DNA from individual progeny plants. Alleles are designated *a–c* with priority given to 'Pomeroy' trifoliate orange

Results

Twenty-one pRLc, 3 gp and 11 pgCit clones revealed RFLPs between grandparental clones for at least one of the tested enzymes. Two clones, pRLc003 and pRLc094, appear to represent moderately repeated sequences because they hybridized to many restriction fragments with all enzymes tested. Only 1 locus of each could be scored. Several clones hybridized to 1 or more monomorphic fragments in addition to the polymorphic fragments reported here. The number of loci corresponding to these clones is unknown. Two clones, pRLc032 and pgCit051 identify 2 loci, *pRLc032a* and *pRLc032b* and *pgCit051a* and *pgCit051b*, respectively. At locus *pRLc032b* only *Citrus* fragments were detected. Too few gp clones have been tested to determine whether cDNA and genomic probes differ in their ability to identify polymorphisms.

Due to the heterozygous nature of the grandparents in this cross, 1:1, 1:2:1, and 1:1:1:1 (Fig. 1) segregation ratios were observed. Segregation of all 8 isozymes, rbcS, and 28 of 37 RFLP loci fit the expected Mendelian ratios. Three and 5 loci deviated significantly at $P<0.05$ and $P<0.01$, respectively, while 1 locus deviated at $P<0.001$ (Table 1). In each case except one, *pgCit051a*, a particular *Citrus* allele is underrepresented.

Two-, three-, and n-point linkage analysis with MAPMAKER and a LOD score of at least 3.0 produced a base map of 31 loci in nine linkage groups. The 1:1 segregating loci were considered linked to one another or to mapped loci if the two-point χ^2 test for linkage showed significant deviation from independent assortment at $P<0.01$ and the maximum likelihood estimate of recombination was less than 30%. In this way, seven 1:1 segregating loci were added to the map. One of these loci (*pRLc053*) was linked to a locus found unlinked by MAPMAKER, allowing this locus (*Idh*) to be added to the map. Thirty-eight out of 46 loci (82.6%) were placed into ten linkage groups. A total of 351 cM lie within the linked loci (Fig. 2).

For those locus pairs with a LOD for linkage greater than 3.0, two-point estimates of recombination from MAPMAKER were always very similar (average difference 1.1 cM) to the maximum likelihood estimates for completely classified (1:1:1:1) data. Therefore, converting data from 1:1:1:1 to 1:2:1 segregation had little effect on recombination estimates. In adding loci with 1:1 segregation to the base map, two-point maximum likelihood estimates from fully classified data were used because loci with 1:1:1:1 segregation provide more information for estimating recombination than loci demon-

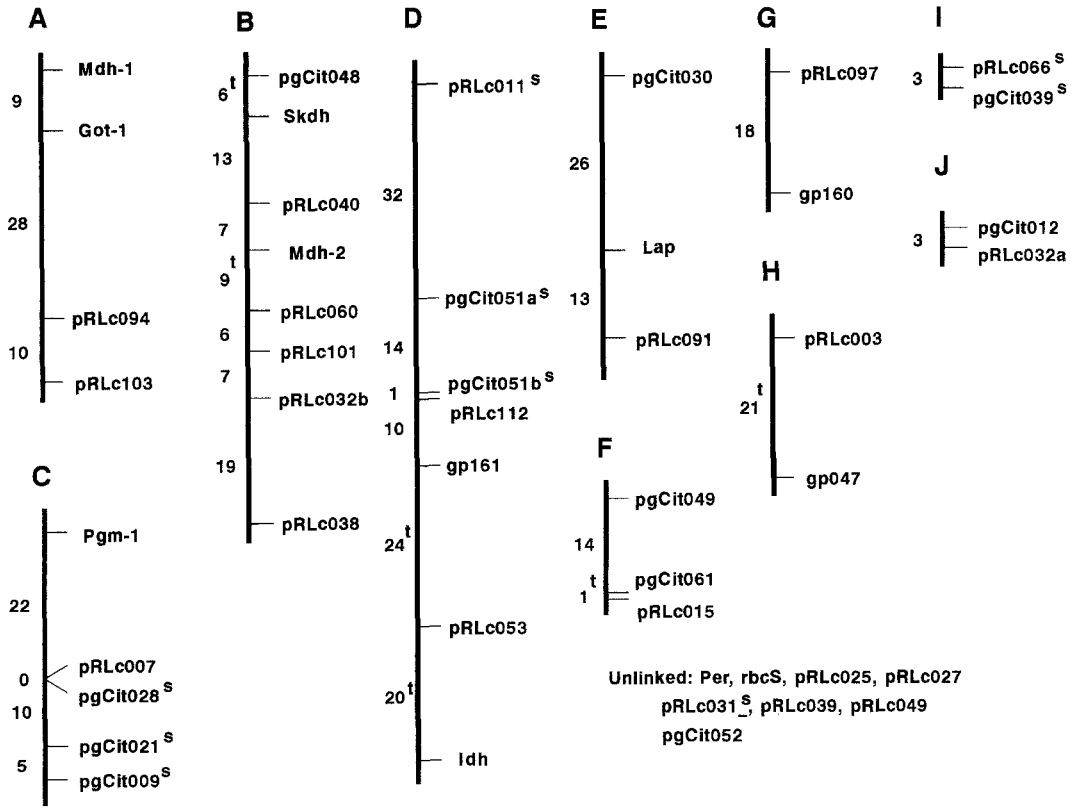

Fig. 2. Linkage map of 38 markers in citrus. Map distances (cM) are on the *left*. [s] designates loci with aberrant segregation, [t] designates two-point map distances between added loci and the nearest MAPMAKER-placed locus

Table 1. Parental genotypes, segregation, and goodness-of-fit tests for RFLP loci showing skewed segregation in the cross of 'Sacaton' citrumelo × 'Troyer' citrange

Locus	Enzyme	Parents		Expected ratio	Observed ratio[a]						χ^2
		Sac	Tro		aa	ab	bb	ac	bc	cc	
pRLc011	*Xba*I	ab	ac	1:1:1:1	15	21	–	5	16	–	9.456*
pRLc031	*Eco*RI	ab	bc	1:1:1:1	–	22	22	8	7	–	14.288**
pRLc066	*Hin*dIII	ab	ab	1:2:1	25	19	6	–	–	–	17.320***
pgCit009	*Eco*RI	ac	bc	1:1:1:1	–	13	–	13	26	8	11.867**
pgCit021	*Eco*RI	ab	ab	1:2:1	13	39	8	–	–	–	6.233*
pgCit028	*Eco*RI	ab	ac	1:1:1:1	16	25	–	11	8	–	11.067*
pgCit039	*Hin*dIII	ab	ab	1:2:1	25	28	7	–	–	–	11.067**
pgCit051a	*Hin*dIII	ab	ab	1:2:1	4	39	10	–	–	–	13.151**
pgCit051b	*Hin*dIII	ac	ab	1:1:1:1	16	4	–	21	10	–	12.765**

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

[a] – designates genotypes not expected or observed for locus

strating 1:2:1 segregation (Allard 1956). The additional information provided by more completely classified loci seems particularly important when testing linkage with the less informative 1:1 loci. For example, the map distance between *Mdh-2* and *pRLc032b* was 14.5 cM using completely classified data, but only 3.5 cM when *pRLc032b* was converted to 1:2:1 segregation.

Discussion

So far, it seems that few repetitive sequences are present in the cDNA- and *Pst*I-derived genomic libraries used in this study. Libraries generated from transcribed sequences contain mostly low- or single-copy genes (Goldberg et al. 1973). Repetitive DNA may make up a sub-

stantial portion of many genomic libraries, but the use of methylation-sensitive restriction enzymes (e.g., *Pst*I) tends to select single-copy regions (Figdore et al. 1988; Helentjaris et al. 1986). Another factor reducing the frequency of repetitive DNA in citrus may be the small genome size (1C = 0.6 pg) (Guerra 1984). The multibanded patterns found when pRLc003 and pRLc094 hybridized to digested citrus DNA suggest that loci *pRLc003* and *pRLc094* may represent members of multigene families.

DNA clones that hybridize to multiple loci indicate that duplications exist in citrus. cDNA clone pRLc032 and genomic clone pgCit051 each identify 2 loci. *pRLc032a* and *pRLc032b* are located on linkage groups J and B, respectively. The lack of a visible fragment in *Poncirus trifoliata* at *pRLc032b* may represent a null allele. The *Citrus* allele may have arisen through a duplication-translocation event in *Citrus* at some point after the genera diverged. Although cytogenetic evidence for translocations has been found in a few *Citrus* species and cultivars (reviewed in Iwamasa and Nito 1988), no evidence exists for their occurrence in *Poncirus* × *Citrus* hybrids. Alternatively, the *Poncirus* allele may consist of one or more small fragments that were not detected. Patterns observed on digests of parental and grandparental clones with *XbaI* seem to support the first hypothesis in that no *Poncirus* allele was detected. *Eco*RI fragment patterns could not be interpreted without segregation analysis. This situation may be clarified by mapping additional loci, which may show that the small linkage group (J) containing *pRLc032a* is linked to B, suggesting an intrachromosomal duplication event. *pgCit051a* and *pgCit051b* are 14 map units apart on linkage group D.

The high percentage of RFLP loci which differ between *Citrus* and *Poncirus* reflect substantial sequence divergence between these genera. Although sexually fairly compatible, *Citrus* and *Poncirus* are isolated by reproductive barriers including apomixis, slightly different flowering times and, in the wild, habitat isolation. Extended asexual propagation allows phenotypically recessive mutations to accumulate, and this, coupled with reproductive isolation, could permit a substantial number of point mutations to accumulate between the two genomes. In maize, insertions and/or deletions are believed to be responsible for many polymorphisms at loci that differ with two or three restriction enzymes out of three (Burr et al. 1983). This argument is less convincing for comparisons between *Citrus* and *Poncirus* because of the greater potential for divergence by point mutation. Determination of the causes of the observed variation in citrus will require more extensive testing and restriction mapping.

Skewed segregation appears to be common in woody perennials (reviewed in Torres et al. 1985). It has also been observed in varying degrees in linkage studies involving wide hybridizations. In interspecific crosses the frequency of RFLP loci with skewed segregation ranged from 22% (Bernatzky and Tanksley 1986) to 100% (Nienhuis et al. 1987). The linkage analysis of the cross of 'Sacaton' and 'Troyer' and the complementary study in Florida (Durham 1990) are, to our knowledge, the first use of intergeneric hybrids to produce a genetic map. The percentage of loci demonstrating aberrant segregation is less than that found in interspecific crosses of other organisms. This suggests that, while these genera are taxonomically distinct at the morphological level, their genomes retain a large amount of structural and functional homology.

The localization of loci with aberrant segregation to three chromosomal regions suggests that these areas contain structural differences and/or genes that effect viability. As a result, estimates of recombination and, thus, map distances between loci with skewed segregation may not be accurate.

On linkage groups C and D, loci showing significantly skewed segregation (*pgCit028* and *pgCit051b*) are closely linked to loci that segregate normally. In both cases, the abberant locus segregates 1:1:1:1 with an excess of one trifoliate/citrus heterozygote class and a deficiency of the other. Such skewed segregation could not be detected at the linked locus because the deficient and excess genotypes are not distinguishable.

Linkage between *Got-1* and *Mdh-1* has been reported in other trifoliate orange hybrids (Torres et al. 1985). Using accumulated data from several small families and two-point linkage analysis, Torres and coworkers (1985) estimated that these loci were 7 cM apart. The present study of a single family with multipoint analysis produced an estimate of 9 cM.

The two-point estimates of recombination determined by MAPMAKER differ only slightly from the maximum likelihood estimates. These differences are thought to be due to the different computational algorithms incorporated into these programs. Also, the multipoint estimates (MAPMAKER) are quite similar (±2 cM) to the two-point estimates except in linkage group B. The two-point estimates between *pRLc032b*, *pRLc060* and *pRLc101* and other members of this linkage group made it very difficult to derive their gene order. Multipoint analysis with MAPMAKER was used to test all possible gene orders. The likelihoods of the two most probable orders were similar. The second most likely order differed in that *pRLc032b* and *pRLc060* were switched. Further mapping and/or a larger progeny should produce a better defined gene order.

The largest linkage group, D, contains 101 cM within the linked markers and could represent a nearly complete chromosome. The other linkage groups range from 67 cM to 3 cM.

In mapping studies in which recombination may be inhibited (e.g., interspecific or intergeneric crosses), it seems particularly appropriate to estimate map length using partial linkage data. Inhibited recombination is suggested by evidence of chromosomal differentiation in *Poncirus* × *Citrus* hybrids such as a higher frequency of rod bivalents (Agarwal 1987) and univalents (Iwamasa 1966) during meiosis than found in *Citrus* × *Citrus* hybrids. This estimate of map length will be useful since hybrids such as those used in this study are commonly used in citrus rootstock breeding.

Estimates of map length according to the method-of-moments estimator of Hulbert et al. (1988) were 1687 cM for a LOD of 2.0, and 1728 cM for a LOD of 3.0. When the equation of Lange and Boehnke (1982) is used and chromosome ends adjusted, approximately 35% of the genome should be linked within 10 cM and 58% within 20 cM of the 46 markers, given a map length of 1700 cM. Recently, Chakravarti et al. (1991) showed that the Hulbert method overestimates map length in comparison to maximum likelihood methods. The extent to which this is true for our data set is not known, but an average chromosome length of almost 200 cM as suggested by this estimate seems unreasonably large in comparison with other plant chromosomes. Although the variance of this estimate is unknown, it is certainly rather large. Estimates of map length based on four random subsamples of 20 loci ranged from 1105 to 2477 cM.

It should be possible to extend this genetic map to include more marker loci. The individual progeny can be vegetatively propagated and should segregate for a number of traits of interest to breeders including resistance to the tristeza virus, Phytophthora root rot, citrus nematode, apomixis, and iron-chlorosis sensitivity. Identification of markers for genes associated with these and other traits should improve the efficiency of citrus rootstock breeding.

Acknowledgements. We thank Drs. G. Jones and R. Shaw for comments on an earlier version of this manuscript, and Dr. L. Walling for technical advice. This work was supported in part by the California Citrus Research Board.

References

Agarwal PK (1987) Cytogenetical investigation in Rutaceae II. Meiotic studies in three intergeneric hybrids of *Citrus*. Cytologia 52:757–760

Allard RW (1956) Formulas and tables to facilitate the calculation of recombination values in heredity. Hilgardia 24:235–278

Bernatzky R, Tanksley SD (1986) Majority of random cDNA clones correspond to single loci in the tomato genome. Mol Gen Genet 203:8–14

Bonierbale MW, Plaisted RL, Tanksley SD (1988) RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095–1103

Burr B, Evola SV, Burr FA, Beckmann JS (1983) The application of restriction fragment length polymorphism to plant breeding. In: Setlow JK, Hollaender A (eds) Genetic engineering: principles and methods, vol 5. Plenum Press, New York, pp 45–49

Cameron JW, Frost HB (1968) Genetics, breeding, and nucellar embryony. In: Reuther W, Webber HJ, Batchelor LD (eds) The citrus industry, vol 2. Univ Calif. Div Agric Sci, University of California Press, pp 325–370

Chakravarti A, Lasher LK, Reefer JE (1991) A maximum likelihood method for estimating genome length using genetic linkage data. Genetics 128:175–182

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Durham RE (1990) Mapping genes involved in freezing tolerance in a backcross of *Citrus* and *Poncirus* using a linkage map of isozymes and restriction fragment length polymorphisms. PhD thesis, University of Florida, Gainesville

Feinberg AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Figdore S, Kennard W, Song K, Slocum M, Osborn T (1988) Assessment of the degree of restriction fragment length polymorphism in *Brassica*. Theor Appl Genet 75:833–840

Gebhardt C, Ritter E, Devener T, Schachtaschabel U, Walkemeier B, Uhrig H, Salamini F (1989) RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor Appl Genet 78:65–75

Goldberg RB, Galau GA, Davidson RJ, Davidson EH (1973) Nonrepetitive DNA sequence representation in sea urchin embryo. Proc Natl Acad Sci USA 70:3516–3520

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263–269

Guerra M dos S (1984) Cytogenetics of Rutaceae. II. Nuclear DNA content. Caryologia 37:219–226

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Havey MJ, Muehlbauer FJ (1989) Linkages between restriction fragment length, isozyme, and morphological markers in lentil. Theor Appl Genet 77:395–401

Helentjaris T (1987) A genetic linkage map for maize based on RFLPs. Trends Genet 3:217–221

Helentjaris T, Slocum M, Wright S, Schaefer A, Nienhuis J (1986) Construction of linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor Appl Genet 72:761–769

Hulbert SH, Ilott TW, Legg EJ, Lincoln SE, Lander ES, Michelmore RW (1988) Genetic analysis of the fungus, *Bremia lactucae*, using restriction fragment length polymorphisms. Genetics 120:947–958

Iwamasa M (1966) Studies on the sterility in the genus *Citrus* with special reference to the seedlessness. Bull Hortic Res Stn Jpn Ser B 6:1–77

Iwamasa M, Nito N (1988) Cytogenetics and the evolution of modern citrus. In: Goren R, Mendel K (eds) Proc 6th Int Citrus Cong. Margraf Scientific Books, Weikersheim, FRG, pp 265–275

Jarrell DC (1991) A genetic map of citrus based on isozymes and RFLPs. MSc thesis, University of California, Riverside

Keim P, Diers BW, Shoemaker RC (1990) Genetic analysis of soybean hard seededness with molecular markers. Theor Appl Genet 79:465–469

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugenics 12:172–175

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lander ES, Green P (1987) Construction of multilocus genetic maps in humans. Proc Natl Acad Sci USA 84:2363–2367

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Lange K, Boehnke M (1982) How many polymorphic genes will it take to span the human genome. Am J Hum Genet 32:842–845

Maniatis ET, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Martin B, Nienhuis J, King B, Schaefer A (1989) Restriction fragment length polymorphisms associated with water use efficiency in tomato. Science 243:1725–1728

McCough SR, Kochert G, Yu ZH, Khush GS, Coffman WR, Tanksley SD (1988) Molecular mapping of rice chromosomes. Theor Appl Genet 76:815–829

Moore GA, Castle WS (1988) Morphological and isozymic analysis of open-pollinated *Citrus* rootstock populations. J Hered 79:59–63

Murray MG, Hoffman LM, Jarvis NP (1983) Improved yield of full-length phaseolin cDNA clones by controlling premature anti-complementary DNA synthesis. Plant Mol Biol 2:75–84

Nienhuis J, Helentjaris T, Slocum M, Ruggero B, Schaefer A (1987) Restriction fragment length polymorphism analysis of loci associated with insect resistance in tomato. Crop Sci 27:797–803

Paterson AH, Lander ES, Hewitt JD, Peterson S, Lincoln SE, Tanksley SD (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721–726

Rawson JRY, Thomas K, Clegg MT (1982) Purification of total cellular DNA from a single plant. Biochem Genet 20:209–219

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 13:7207–7221

Roose ML (1988) Isozymes and DNA restriction fragment length polymorphisms in citrus breeding and systematics. In: Goren R, Mendel K (eds) Proc 6th Int Citrus Cong. Margraf Scientific Books, Weikersheim, FRG, pp 155–165

Sarfatti M, Katan J, Fluhr R, Zamir D (1989) An RFLP marker in tomato linked to the *Fusarium oxysporum* resistance gene *I2*. Theor Appl Genet 78:755–759

Silflow CD, Hammett JR, Key JL (1979) Sequence complexity of polyadenylated ribonucleic acid from soybean suspension culture cells. Biochemistry 18:2725–2731

Soller M, Beckmann J (1983) Genetic polymorphism in varietal identification and genetic improvement. Theor Appl Genet 6:25–33

Soost RK, Cameron JW (1975) Citrus. In: Janick J, Moore JN (eds) Advances in fruit breeding. Purdue University Press, West Lafayette, Ind., pp 507–540

Soost RK, Williams TE, Torres AM (1980) Identification of nucellar and zygotic seedlings of citrus with leaf isozymes. HortScience 15:728–729

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Swingle WT, Reece PC (1967) The botany of *Citrus* and its wild relatives. In: Reuther W, Webber HJ, Batchelor LD (eds) The citrus industry, vol 1, revised edn. Div Agric Sci, University of California, Berkeley, pp 190–430

Tanksley SD (1983) Molecular markers in plant breeding. Plant Mol Biol Rep 1:3–8

Tanksley SD, Hewitt J (1988) Use of molecular markers in breeding for soluble solids content in tomato – a re-examination. Theor Appl Genet 75:811–823

Tanksley SD, Rick C (1980) Isozymic gene linkage map of the tomato: application in genetics and breeding. Theor Appl Genet 57:161–170

Tanksley SD, Medina-Filho H, Rick C (1982) Use of naturally occurring enzyme variation to detect and map genes controlling quantitative traits in an interspecific backcross of tomato. Heredity 49:11–25

Tanksley SD, Bernatzky R, Lapitan NL, Prince JP (1988) Conservation of gene repertoire but not gene order in pepper and tomato. Proc Natl Acad Sci USA 85:6419–6423

Torres AM (1983) Fruit trees. In: Tanksley SD, Orton TJ (eds) Isozymes in plant genetics and breeding. Elsevier Scientific, Amsterdam, pp 401–421

Torres AM, Soost RK, Mau-Lastovicka T (1982) Citrus isozymes: genetics and distinguishing nucellar from zygotic seedlings. J Hered 73:335–339

Torres AM, Mau-Lastovicka T, Williams TE, Soost RK (1985) Segregation distortion and linkage of *Citrus* and *Poncirus* isozyme genes. J Hered 76:289–294

Xiang C, Roose ML (1988) Frequency and characteristics of nucellar and zygotic seedlings in 12 citrus rootstocks. Sci Hortic 37:47–59

Young ND, Tanksley SD (1989) RFLP analysis of the size of chromosomal segments retained around the *Tm-2* locus of tomato during backcross breeding. Theor Appl Genet 77:353–359