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Linkage of restriction fragment length polymorphisms and isozymes in *Citrus*

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Summary. Genetic linkage analysis was performed using two segregating populations of citrus. One population arose from an intergeneric backcross of *Citrus grandis* (L.) Osb. cv 'Thong Dee' and *Poncirus trifoliata* (L.) Raf. cv 'Pomeroy', using the former as the recurrent (female) parent. The other population came from an interspecific backcross of *C. reticulata* Blanco cv 'Clementine' and *C.* × *paradisi* Macf. cv 'Duncan', using the former as the recurrent (male) parent. A total of 11 isozyme and 58 restriction fragment length polymorphisms were found to segregate in a monogenic fashion in one or both populations. Linkage analysis revealed that 62 of the loci examined mapped to 11 linkage groups, while 7 loci segregated independently from all other markers. Gene order was highly conserved between the maps generated from the two divergent segregating populations. Possible applications of the use of such maps in tree fruit breeding are discussed.

Key words: Genetic map – Molecular markers – RFLP – Fruit breeding – *Citrus* spp.

Introduction

Genetic analysis of perennial fruit species has been hindered by several factors including large plant size, long juvenility periods, self- and cross-incompatibility, inbreeding depression, and apomixis (Janick and Moore 1975; Moore and Janick 1983). Genetic linkage maps have not been described for any tree fruit crop because few Mendelian markers are known in these species. The recent development and application of isozyme techniques for many plant species, including fruit crops, have increased the number of genetic markers available (Torres 1983, 1989; Weeden 1989). However, the small number of isozyme loci analyzed in most fruit crops precludes their use as the sole basis for the creation of genetic linkage maps. Linkage relationships among loci encoding biologically or economically important traits remain virtually unknown in these species.

Restriction fragment length polymorphisms (RFLPs) are being extensively used to create linkage maps for plants and animals. RFLPs were initially used to define further the already extensive maps available for maize and tomato (Helentjaris et al. 1986; Bernatzky and Tanksley 1986b). More recently, RFLPs have been used to initiate linkage map construction for plants where few prior linkage relationships had been determined; e.g., lentil (Havey and Muehlbauer 1989), lettuce (Landry et al. 1987), pepper (Tanksley et al. 1988), and potato (Bonierbale et al. 1988; Gebhardt et al. 1989).

Citrus, in many respects, is the most attractive fruit tree crop for conducting linkage analysis. It is a diploid with a relatively low haploid chromosome number, n = 9 (Soost and Cameron 1975), and small genome, 1C = 0.62 pg (Guerra 1984). The genus is highly polymorphic, and interspecific as well as some intergeneric hybrids are easily made (Barrett 1977, 1985). Genetic analyses of several isozyme systems have been reported (Torres et al. 1978, 1982), and initial studies have revealed two linkage groups comprised of five isozyme loci (Torres et al. 1985). Further, citrus is the most widely produced fruit crop in the world, but cultivar improvement programs have been hindered by factors of reproductive biology (sterility, incompatibility, nucellar embryony, juvenility) and the lack of information on the nature and mode of inheritance of economically significant traits. A linkage map for citrus should provide a

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useful tool to expedite the development of improved cultivars. The goals of this research were to investigate the potential of combining isozyme and RFLP analysis to create a linkage map for citrus and to determine if gene order was conserved among divergent citrus types.

Material and methods

Plant material

Two BC_1 populations were constructed for genetic analyses. One population (CXP) resulted from an intergeneric cross of two divergent kinds of citrus – 'Thong Dee' pummelo (*C. grandis* (L.) Osb.) and 'Pomeroy' trifoliate orange (*Poncirus trifoliata* (L.) Raf.). 'Pomeroy' was used as the donor parent, and the intergeneric hybrid (selection 17–40, pollen and plant material obtained from H. C. Barrett, USDA, Fl.) served as the male parent. Pollinations of 'Thong Dee' were made at the Florida Citrus Arboretum, Division of Plant Industries, Winter Haven, Fl. The other population (CXC) resulted from an interspecific cross of two more closely related kinds of citrus – 'Clementine' mandarin (*C. reticulata* Blanco) and 'Duncan' grapefruit (*C.* × *paradisi* Macf.). 'Duncan' served as the donor parent, and the cross was made using the interspecific hybrid (selection LB 1-21) as the female parent. Sixty-five progeny from each population were selected at random and used for genetic analyses.

Isozyme analysis

Isozyme analysis was performed using 13 enzyme staining systems and previously described procedures (Durham et al. 1987; Moore and Castle 1988). The enzymes analyzed included acid phosphatase (ACP, EC 3.1.3.2), alkaline phosphatase (AKP, EC 3.1.3.1), glutamate oxaloacetate transaminase (GOT, synonymous with aspartate aminotransferase, EC 2.6.1.1), isocitrate dehydrogenase (IDH, EC 1.1.1.42), leucine amino peptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.4.0), peroxidase (PER, EC 1.11.1.7), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), shikimate dehydrogenase (SkDH, EC 1.1.1.25), and superoxide dismutase (SOD, EC 1.15.1.1). These isozymes cumulatively represent at least 20 loci (Torres 1983; Roose 1988; Moore and Gmitter unpublished data).

Rubisco SSU analysis

For analysis of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU), leaf proteins were extracted into buffer (125 m*M* TRIS-HCl pH 6.8, 10% 2-mercaptoethanol, 4.6% SDS, and 20% glycerol) and electrophoresed in 10% or 15% polyacrylamide gels containing 1% SDS, with the buffer solutions as described by Laemmli (1970). For Western analysis, proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Richmond, Calif.). The immunoassay was performed using standard procedures (Harlow and Lane 1988) with a polyclonal antibody raised against *Lemna* Rubisco SSU made available by B. O. Kohorn, Duke University, Durham, N.C. Bound primary antibodies were detected using an alkaline phosphatase conjugated secondary antibody (Protoblot Immunoscreening System, Promega Bioteck, Madison, Wis.).

Isolation, digestion, and blotting of citrus DNA

DNA was isolated from citrus leaves using the method of Dellaporta et al. (1983) except that after the first precipitation in isopropanol, the redissolved DNA was extracted with phenol, phenol/chloroform, and chloroform (Maniatis et al. 1982), then precipitated with ethanol and redissolved in TE (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0). Digestion of DNA with restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, or *Pst*I was according to manufacturers' instructions (Bethesda Research Laboratories, Gaithersburg, Md., or Boehringer Mannheim Biochemicals (BMB), Indianapolis, Ind.). Electrophoresis of the digested DNA (1–2 μg/lane) was performed in 1.0% agarose gels in 1 × TPE (80 m*M* TRIS-phosphate, 2 m*M* EDTA), and the DNA was transferred to nylon membranes (Hybond-N) according to the manufacturer's instructions (Amersham, Arlington Heights, Del.).

Sources of probes for RFLP analysis

The probe designated pcPt001 was from a cDNA library constructed from mRNA isolated from cold-acclimated 'Pomeroy' trifoliate orange (Durham 1990). The mRNA was separated from total RNA using hybond MAP paper (Amersham), and cDNA synthesis was with a kit from BMB. The cDNA was ligated to *Eco*RI 8-mer linkers (BMB), ligated into plasmid pTZ18R, and used to transform *E. coli* strain *TB1* according to Hanahan (1985). The library consisted of approximately 1,000 clones; the cloning efficiency was 3×10^5 clones/μg of insert. The probes designated pgCit were from a *Pst*I genomic library of 'Temple' tangor (*Citrus reticulata* Blanco × *C. sinensis* L. Osb.) (Liou 1990). This genomic library was constructed with DNA isolated from pelleted nuclei and digested with *Pst*I. Genomic fragments were separated by sucrose gradient centrifugation, and fragments approximately 500–2,500 base pairs in length were ligated into plasmid pTZ18R and used to transform *E. coli* strain *TB1*. The library consisted of 970 clones; the cloning efficiency was approximately 1.4×10^5 clones/μg of insert. Probes designated pRLc were from a cDNA library of mRNA isolated from leaves of rough lemon (*C. jambhiri* Lush) and were provided by M. L. Roose, University of California, Riverside.

Radioactive labeling and hybridization of probes

Cloned inserts used as probes were isolated using Geneclean (Bio 101, La Jolla, Calif.) following digestion with appropriate restriction enzymes and electrophoresis in 1% agarose, 1 × TPE. Probes were labeled using a random primer labeling kit (BMB). Typical reactions included 100 ng of isolated insert and 40–50 μCi α-[^{32}P]dCTP (New England Nuclear, Boston, Mass.) resulting in specific activities of $\geq 10^8$ DPM/μg of insert. Unicorporated label was separated from probes by gravity flow exclusion chromatography through Sephadex G-50-50 (Sigma) as described in Maniatis et al. (1982). Blots to be probed were prehybridized (65 °C, 2–24 h) and hybridized (65 °C, 18–24 h) according to Church and Gilbert (1984). Following hybridization, blots were washed 2 times for 30 min each at 65 °C in 2 × SSC (1 × SSC is 150 m*M* NaCl, 15 m*M* NaCitrate, pH 7.0), 0.1% SDS; this was followed by two washes for 30 min each at 65 °C in 0.2 × SSC, 0.1% SDS. Autoradiography was performed with Kodak X-omat AR X-ray film and intensifying screens (Dupont Cronex Lightning-Plus) at −80 °C for 1–4 days. Probe removal was achieved by soaking membranes in 0.4 *N* NaOH for 30 min at 45 °C, then neutralizing in 0.2 *M* TRIS-HCl pH 7.2, 0.1 × SSC, 0.1% SDS for 30 min at 45 °C. Blots were routinely reused six to eight times.

Linkage analysis of BC_1 segregation

The linkage map of the CXP population was constructed using MAPMAKER (Lander et al. 1987; Whitehead Institute, Cambridge, Mass.) with default linkage criteria of LOD ≤ 2.0, re-

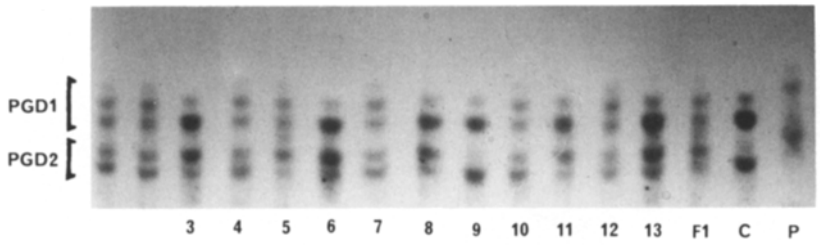

Fig. 1. Banding patterns observed for isozymes of 6-phosphogluconate dehydrogenase in the CXP backcross population of citrus. Lanes include extracts from *P. trifoliata* (*P*), *C. grandis* (*C*), their F_1 hybrid (*F1*), and backcross progeny (*lanes* 1–13). Genotypes of backcross progeny for *Pgd1*/*Pgd2* are: cp/cp in lanes 1, 2, 4, 5, 7, 10 and 12; cc/cp in lanes 3, 6, 8, 11, and 13; and cc/cc in lane 9. cc = homozygous for *C. grandis* allele, cp = heterozygous for *C. grandis* and *P. trifoliata* alleles. The origin of migration is at the *bottom* of the photograph, and band migration is towards the anode

combination fraction ≤ 0.4, and the Kosambi (1944) mapping function. Because segregation in the CXC population involved both backcross (1:1) and F_2 (1:2:1) ratios, the entire set of markers was processed through the LINKAGE-1 computer program for linkage analysis (Suiter et al. 1983) because MAPMAKER could not accommodate mixed types of segregation. The LINKAGE-1 program was capable of performing only two-point analysis, so this linkage map was deduced as the best fit to these values following the principle of the three-point-cross method (Suzuki et al. 1981).

Results and discussion

Isozyme segregation

Of the 13 enzyme staining systems investigated, 7 (GOT, ME, MDH, IDH, PGD, PGM, and SkDH) gave well-resolved banding patterns and exhibited genetically interpretable polymorphism within the CXP population, while 6 systems (ACP, GOT, IDH, PGM, PGI, SDH) were useful in the CXC population. The segregation data for these isozymes are presented in Table 1. Locus and allele nomenclature, where applicable, were according to Torres et al. (1978, 1982, 1985). Segregation of PGD was also apparent in the CXP population and is illustrated in Fig. 1. Segregation of PGD has not been reported previously in citrus. Two zones of activity were evident; the faster migrating zone was designated PGD1, and the slower zone PGD2. *C. grandis* exhibited a single dark band and a fainter, faster-migrating band at both the PGD1 and PGD2 regions, while the F_1 exhibited three bands at PGD1 and two bands at PGD2. The banding pattern for *P. trifoliata* was unclear. However, the BC progeny all exhibited banding patterns that were similar to either *C. grandis* or the F_1 hybrid at either PGD1 or PGD2. This would be expected if separate loci were responsible for the polymorphism observed for PGD1 and PGD2; *C. grandis* was homozygous at both loci, and the F_1 was heterozygous at both loci. These loci were designated *Pgd1* and *Pgd2* in accordance with previous nomenclature for citrus (Torres et al. 1978, 1982, 1985).

Table 1. Monogenic segregation of isozyme loci in two backcross populations of citrus

Locus	Parental phenotype ♀	Parental phenotype ♂	Test ratio	Progeny segregation	Chi-square[b]
			Population CXP		
Got1	SF	SP	1:1:1:1	20 SS:29 FS:7 FP:7 SP[a]	22.02**
Idh	II	FI	1:1	32 II:31 FI	0.02
Mdh2	FF	SF	1:1	47 FF:16 FS	12.94**
Me1	II	FI	1:1	45 II:18 FI	11.57**
Pgd1	CC	CP	1:1	36 CC:27 CP	1.29
Pgd2	CC	CP	1:1	22 CC:40 CP	5.23*
Pgm1	SS	SM	1:1	32 SS:31 SM	0.02
Skdh	SS	FS	1:1	39 SS:24 FS	3.57
Ssu	CC	CP	1:1	32 CC:26 CP	0.62
			Population CXC		
Acp	FM	MM	1:1	33 FM:28 MM	0.41
Got1	FS	SS	1:1	26 FS:39 SS	2.60
Idh	FF	FM	1:1	38 FF:23 FM	3.69
Pgi	FS	FS	1:2:1	16 FF:36 FS:12 SS	1.50
Pgm1	IS	IF	1:1:1:1	22 IF:11 SF:13 II:19 IS	4.85
Skdh	IS	II	1:1	31 II:34 IS	0.14

[a] For linkage analysis the segregation data for *Got1* in population CXP was recorded to reflect only the segregation of the donor male allele in the progeny. The resulting segregation ratio was 49 FS + SS:14 FP + SP with a chi-square of 19.44**

[b] * and ** indicate significant deviation from expected 1:1 ratio at $P = 0.05$ and 0.01, respectively

As demonstrated by progeny analysis (Fig. 1, Table 1), both *Pgd1* and *Pgd2* appear to segregate as single loci. However, because the segregation of *Pgd2* exhibited significant skewing ($P \leq 0.05$) from the expected 1:1 ratio, additional segregating families need to be analyzed to confirm this interpretation of the data. Linkage analysis revealed that these loci are linked by a recombination distance of 26.4 cM.

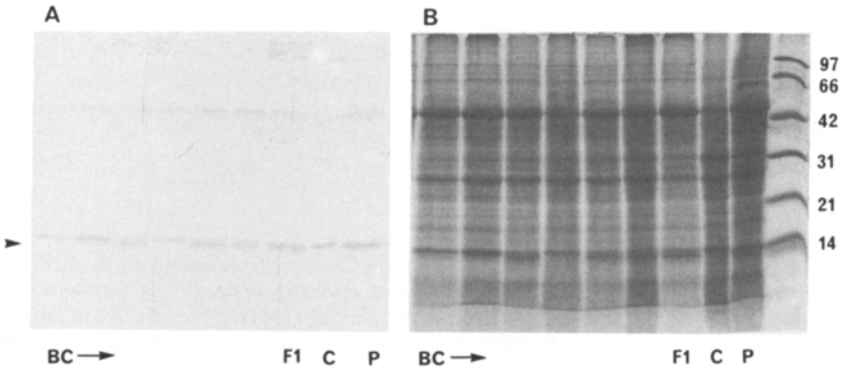

Fig. 2 A–B. Western blot analysis and total protein-stained gel revealing the segregation of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (SSU) in the CXP backcross population. **A** Western blot analysis demonstrating immuno-crossreactivity of a 14-kDa protein (*arrow*) to an antibody raised against *Lemna* SSU. **B** Coomassie-stained gel identical to **A**. Lanes include *P. trifoliata* (*P*), *C. grandis* (*C*), their F_1 hybrid (*F1*), and backcross progeny (*BC*). Genotypes of backcross progeny for *Ssu* include, from *left to right* in both **A** and **B**, cc, cc, cp, cc, cp, and cp. cc = homozygous for *C. grandis* allele, cp = heterozygous for *C. grandis* and *P. trifoliata* alleles. Molecular weight standards (*right-hand lane*) are expressed in kDa

SSU segregation

Polymorphism in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU) has been previously reported in citrus. Handa et al. (1986) were able to distinguish four forms of SSU in various *Citrus* species and related genera using isoelectric focusing (no individual plants had more than two forms of the enzyme). *P. trifoliata* exhibited a unique form of the enzyme. Roose et al. (1988) reported that in crosses between *Citrus* and *Poncirus* the polymorphism in SSU exhibited monogenic inheritance based on data collected at both the protein and DNA level. Polymorphism between *C. grandis* and *P. trifoliata* was also observed in the present study in a protein of approximately 15 kDa (Fig. 2), presumably SSU, which segregated in a 1:1 ratio (Table 1) in the CXP population. The identity of this protein as SSU was supported by its crossreactivity to a polyclonal antibody raised against *Lemna* SSU (Fig. 2). Both *C. grandis* and *P. trifoliata* exhibited a single band with the *Citrus* band migrating slightly more slowly than the *Poncirus* band (Fig. 2). The F_1 hybrid possessed both bands, and the BC progeny were identical to either the F_1 hybrid two-banded phenotype or the single band characteristic of *C. grandis*. Therefore, segregation of alleles at a single locus, designated *Ssu*, appears to control the polymorphism observed between *C. grandis* and *P. trifoliata* for SSU.

Probe selection and RFLP analysis

In an initial study, Southern blots containing DNA isolated from the parents of the CXP population, *P. trifoliata, C. grandis,* and the F_1 hybrid, were made by digesting the DNAs with one of several enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, or *Pst*I). The resulting blots were hybridized with randomly selected clones from both the cDNA (pcPt) and genomic (pcCit) libraries to determine if one or the other library might be more efficient at detecting polymorphisms. The cDNA clones, in general, exhibited weaker hybridization signals than genomic clones, in agreement with previous reports (Helentjaris et al. 1986). Furthermore, most of the cDNA clones exhibiting a strong hybridization signal produced unexpected banding patterns suggestive of multiple copy or highly repetitive sequences (i.e., the hybridization signal was dispersed throughout the lanes, or the hybridization signal was several orders of magnitude greater than would be expected from a single or low copy sequence). When polymorphisms were detected between the two species, the banding patterns were complex. For these reasons, only 1 of 21 clones (< 5%) from the pcPt library was useful as a probe for progreny RFLP analysis. The clones from a second cDNA library, designated pRLc, had been previously selected as being useful to detect polymorphisms among different *Citrus* species (Roose et al. 1988). Of 20 pRLc clones tested, 14 (70%) proved useful for progeny RFLP screening in the CXP population and 5 (25%) were useful in the CXC population.

The *Pst*I genomic library proved to be a much better source of useful probes. In other species, *Pst*I has proven advantageous for genomic library construction because the enzyme does not cleave at restriction sites that are methylated. Because regions of the genome that are actively being transcribed are usually undermethylated (Burr et al. 1988), *Pst*I genomic libraries tend to be enriched for single or low copy number sequences suitable for RFLP analysis. In initial studies using the pgCit clones with the CXP population, 11 of 18 probes (61%) detected a polymorphism between the two genera that was potentially useful for progeny segregation analysis. Banding patterns produced by these genomic probes were less complex (fewer bands per lane) and thus more

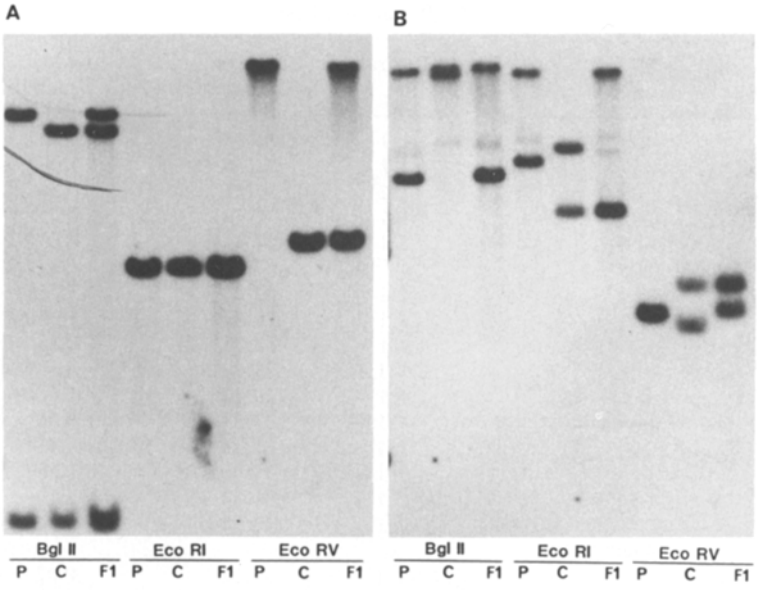

Fig. 3A, B. Southern blots of parental genotypes used to select useful clone/restriction enzyme combinations for detecting restriction fragment length polymorphisms (RFLPs) in the CXP backcross population of citrus. Lanes are designated as containing DNA restricted with enzyme *Bgl*II, *Eco*RI, or *Eco*RV, isolated from *P. trifoliata* (*P*), *C. grandis* (*C*), or their F_1 hybrid (*F1*). **Panel A** was probed with clone pgCit035, which detected a polymorphism when the DNA was digested with either *Bgl*II or *Eco*RV, but not when digested with *Eco*RI. **Panel B** was probed with clone pgCit045S, which detected a polymorphism with any of the three enzymes. In panel A, both *P. trifoliata* and *C. grandis* are homozygous for the RFLP while in panel B, *C. grandis* is heterozygous for the RFLP with any enzyme while *P. trifoliata* is heterozygous when restricted with either *Bgl*II or *Eco*RI

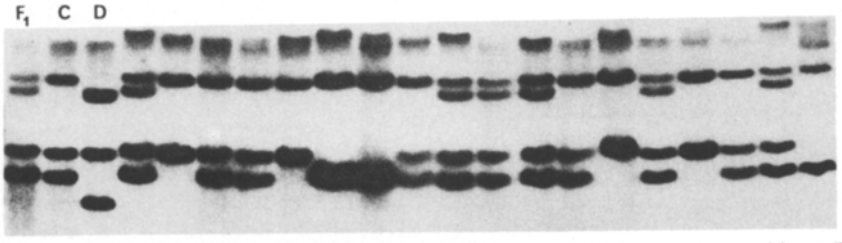

Fig. 4. Southern analysis segregation of restriction fragment length polymorphisms (RFLPs) in the CXC backcross population of citrus. Lanes contain DNA restricted with *Bgl*II. The blot was probed with two clones. The *upper set* of segregating bands was produced by hybridization with clone pgCit035, while the *lower bands* were produced by hybridization with pgCit053. *Lane 1* (*F1*) contains DNA from the F_1 hybrid of *C. reticulata* cv 'Clementine' (*lane 2, C*) and *C.* × *paradisi* cv 'Duncan' (*lane 3, D*), while the remaining lanes contain DNA from segregating progeny from the backcross of the F_1 and 'Clementine'. The progeny segregated in a 1:1 ratio for the polymorphism detected by pgCit035 and in a 1:2:1 ratio for that detected by pgCit053

easily interpreted than patterns produced by cDNA probes. As expected, a few probes from the genomic library hybridized to repetitive sequences or sequences exhibiting maternal inheritance and were not further characterized.

Following the initial screening of 21 pcPt clones and 18 pgCit clones, further probe selection was only from the pgCit genomic library because of the higher percentage of useful polymorphisms detected by these probes. Furthermore, in the initial screening it was determined that all polymorphisms detected in the CXP population were found when the DNA was restricted with either *Bgl*II, *Eco*RI, or *Eco*RV. Therefore, additional probe selections involved only these enzymes (Fig. 3). Similar studies were performed using the parents of the CXC population with similar results.

Segregation patterns and distorted segregation ratios

When selected clones were used to screen the two segregating populations, several types of segregation were observed (Table 2; Fig. 4); this was also true of isozyme alleles (Table 1). In the CXP population, backcross-type segregation (i.e., 1:1) predominated because the recurrent parent of this population, *C. grandis,* was homozygous for a majority of the markers analyzed. However, 7 of the 48 RFLP markers analyzed in this population were heterozygous in *C. grandis,* which resulted in allelic combinations other than those usually found in a backcross population. The donor allele contributed by *P. trifoliata* could always be distinguished from either of the *C. grandis* alleles, allowing the segregation data to be simplified by following only the segregation of the donor

Table 2. Monogenic segregation of restriction fragment length polymorphisms in two backcross populations of citrus

Locus	Population CXP			Population CXC		
	Test ratio	Progeny segregation	Chi-squared[c]	Test ratio	Progeny segregation	Chi-squared
pgCit001	–[a]	–	–	1:1	32:33	0.02
pgCit005	(1:1)[b]	40:23	4.59*	1:1	28:36	1.00
pgCit009	1:1	36:28	1.00	1:1	30:35	0.54
pgCit010	1:1	31:32	0.02	1:1	34:30	0.25
pgCit011	(1:1)	27:37	1.56	1:1	24:41	4.45*
pgCit012	1:1	29:36	0.75	–	–	–
pgCit015	1:1	31:30	0.02	1:1	42:23	5.55*
pgCit016	–	–	–	1:1	28:37	1.25
pgCit017	1:1	42:22	6.25*	1:1	27:31	0.28
pgCit019	1:1	33:31	0.06	1:1:1:1	25:9:18:13	8.78*
pgCit020	–	–	–	1:1	40:20	6.67*
pgCit021	1:1	37:27	1.56	1:1	33:31	0.06
pgCit025	–	–	–	1:1	41:24	4.45*
pgCit026	–	–	–	1:1	35:30	0.38
pgCit027	1:1	37:27	1.56	1:1	31:34	0.14
pgCit028	1:1	27:32	0.51	1:1	32:27	0.42
pgCit030	1:1	18:24	9.60*	–	–	–
pgCit031S	–	–	–	1:1	31:32	0.02
pgCit035	1:1	37:24	2.77	1:1	32:33	0.02
pgCit037	1:1	37:28	1.25	1:1	26:32	0.62
pgCit039	1:1	35:27	1.03	–	–	–
pgCit041	–	–	–	1:1	31:34	0.14
pgCit042	1:1	43:18	10.25*	–	–	–
pgCit045	1:1	43:21	7.56*	–	–	–
pgCit046	–	–	–	1:1	34:26	0.30
pgCit048	1:1	39:23	4.13*	1:1	35:30	0.54
pgCit049	(1:1)	40:18	8.35*	1:1	32:33	0.02
pgCit051	1:1	42:22	6.25*	–	–	–
pgCit052	(1:1)	27:38	1.86	–	–	–
pgCit053	–	–	–	1:2:1	10:36:19	3.25
pgCit054S	(1:1)	37:23	3.27	1:1	31:34	0.14
pgCit054L	(1:1)	29:31	0.67	1:1	33:32	0.02
pgCit056	1:1	32:30	0.07	1:1	41:24	4.45*
pgCit057L	1:1	33:31	0.06	–	–	–
pgCit061	1:1	38:27	1.86	–	–	–
pgCit062	1:1	32:29	0.15	1:1	48:17	14.78*
pgCit063	1:1	27:30	0.16	–	–	–
pgCit064	1:1	39:17	8.64*	–	–	–
pgCit065	1:1	40:24	4.00	–	–	–
pgCit069	1:1	33:32	0.02	1:1	26:36	1.16
pgCit071	1:1	35:27	1.03	1:1	51:14	21.06*
pgCit074	1:1	40:16	10.29*	1:1	35:29	0.56
pcPt001	(1:1)	38:26	2.25	1:1	27:38	1.86
pRLc24	1:1	32:30	0.07	1:1	42:23	5.55*
pRLc25	1:1	32:30	0.07	–	–	–
pRLc27L	–	–	–	1:2:1	24:31:10	6.17*
pRLc31	1:1	25:23	0.08	–	–	–
pRLc32	1:1	35:22	2.97	1:2:1	9:56:0	36.48*
pRLc38	1:1	40:22	5.23*	–	–	–
pRLc39	1:1	32:30	0.07	–	–	–
pRLc40	1:1	40:22	5.23*	–	–	–
pRLc41	1:1	29:32	0.15	–	–	–
pRLc49	1:1	42:21	7.00*	–	–	–
pRLc60	1:1	49:15	18.06*	–	–	–
pRLc66L	1:1	37:27	1.56	–	–	–
pRLc89	1:1	35:27	1.03	1:2:1	11:43:11	21.38*
pRLc91	1:1	22:41	5.73*	1:2:1	19:31:15	0.63
pRLc94	1:1	21:42	7.00*	–	–	–

[a] indicates data not taken for this locus in this population
[b] (1:1) indicates that the data were transformed from a 1:1:1:1 ratio to fit a 1:1 ratio; see text and footnote to Table 1 for details
[c] *indicates deviation from expected ratio at $P=0.05$ level of significance

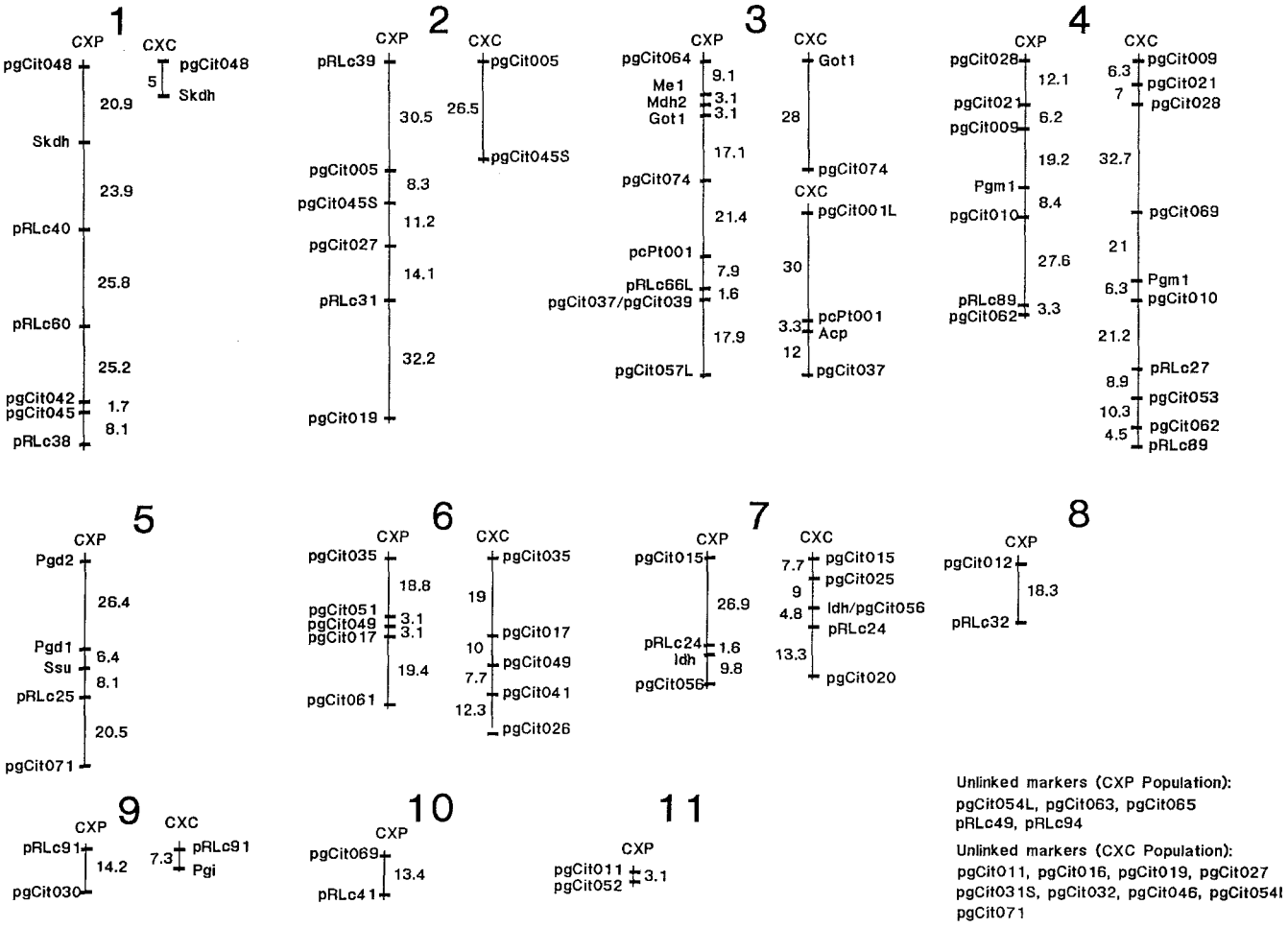

Fig. 5. Linkage map deduced from segregation data of two backcross populations of citrus. Individual linkage groups are designated by *large, bold-faced numbers,* and each linkage group originated from either the CXP or CXC population. Locus names and map distances (in cM) between adjacient markers are listed for each linkage group. Certain regions of the CXP linkage groups were ambiguous as to precise locus order. The precise orders resulting in the highest LOD scores using MAPMAKER are given but in a few cases, the LOD scores between several combinations of loci were very similar (≤ 1 LOD score difference). For example, in linkage group 1, the precise order of markers Skdh and pgCit048 in relation to pRLc40 and the precise order of markers pgCit042 and pgCit045 in relation to marker pRLc38 and pgCit062 in relation to pgCit010 could not be determined, nor could the precise order of markers pRLc24 and IDH of linkage group 7 be determined. Also, in the CXC linkage group 4, marker pRLc32 was linked to pRLc27 and pgCit010 but without evidence of linkage to other loci, and marker pgCit016 was linked to several markers but without good fit using the three-point-cross method

allele. An example of this approach can be found for isozyme data in Table 1. Segregation was more complex in the CXC population, with segregation ratios of 1:2:1 at some loci (Tables 1 and 2, Fig. 4). Therefore, it was not always possible to simplify segregation data as previously described. For this reason, the data were not transformed prior to linkage analysis but were analyzed as mixed type segregation using the LINKAGE-1 program (Suiter et al. 1983).

A large proportion of the markers analyzed in this study exhibited distorted segregation ratios (Tables 1, 2, 3). In the CXP population 37% of the markers exhibited skewed segregation while 29% of the markers in the CXC population exhibited skewing (Table 3). This was not surprising since deviations from expected ratios have been previously reported for molecular markers, especially those analyzed in wide crosses (Weeden 1989). In fact, Torres et al. (1985) observed skewed ratios for as many as seven of eight loci that they examined in certain families of a cross of *C. grandis* and *P. trifoliata*. Skewedness in segregation ratios of molecular markers is not thought to result from pleiotropy or epistasis, but to reflect abnormal segregation (Weeden 1989). Zamir and Tadmore (1986) suggested that such distorted ratios may result from linkage to genes exposed to directional selection at either pre- or post-zygotic stages of development. Therefore, skewedness does not prevent the use of such loci in mapping studies.

Table 3. Segregation distortion of polymorphic loci in two backcross populations of citrus

Type of marker	CXP population	CXC population
Isozyme	4 of 9 (44%)	0 of 6
RFLP-cDNA clones	6 of 15 (40%)	4 of 6 (67%)
RFLP-genomic DNA clones	11 of 33 (33%)	8 of 30 (27%)
Total	21 of 57 (37%)	12 of 42 (29%)

Linkage maps

Analysis of the loci segregating in the CXP population revealed that 52 of these loci fell into 11 linkage groups, while 5 loci remained unassigned to any linkage group (Fig. 5). The total length of the map illustrated in Fig. 5 is 553 cM with the distance between markers ranging from 0 to 32.2 cM (mean distance of 13.5 cM). Analysis of all pairwise combinations of loci segregating in the CXC population revealed that 32 loci fell into 8 linkage groups, while 9 loci remained unlinked (Fig. 5). The total map distance of the linkage groups constructed using the CXC population was 314 cM with the distance between markers ranging from 0 to 32.7 cM (mean distance of 11.6 cM).

When the maps constructed using the two populations were compared, very few discrepancies in locus order were observed. The discrepancies that were apparent were mostly in regions of the CXP population map where precise gene order was ambiguous (see caption to Fig. 5). This conservation of locus order was not surprising; most species of *Citrus* are interfertile, and interspecific hybrids are usually fertile, implying that genome organization between species must be conserved. However, subtle differences between genomes may exist because a large proportion of loci in both populations exhibited skewed segregation ratios, and the loci that exhibited skewing were different in the two populations (Table 2).

Torres et al. (1985) have reported two linkage groups in citrus, *Mdh2*-17 cM-*Me01*-27 cM-*Me02* and *Got1*-7 cM-*Mdh1*. In the present study, only one region of activity could be detected for ME, and it appeared to correspond to the locus described by Torres et al. (1985) as *Me01*. *Me1* was linked to an MDH locus and a GOT locus. Using the nomenclature designated by Torres, the MDH locus was designated *Mdh2; Mdh1* did not segregate in this population. Likewise, the GOT locus analyzed was designated *Got1*, while banding patterns were unclear in the GOT2 region. The linkage relationship among these loci is *Me1*-3.1 cM-*Mdh2*-3.1 cM-*Got1*. One reason for the difference between these results and those of Torres et al. (1985) may be that certain isozymes of *Mdh1* and *Mdh2* overlap in migration rates such that scoring for these loci is not always straight-forward. However, scoring was simplified in this study because *Mdh1* did not segregate. Also, resolution of the second GOT and ME loci has never been clear in the laboratory where these analyses were performed (Moore, unpublished data), possibly because of differences in techniques used for isozyme analysis.

Conclusions

This study is the first to demonstrate the potential of combining RFLP and isozyme analyses for creating a genetic map for a tree fruit species. It is obvious that the genome has not been saturated with markers, since the number of linkage groups reported is greater than the number of haploid chromosomes of citrus (n=9). It is likely that several of the smaller linkage groups will converge or join with larger linkage groups with the analysis of additional markers. The fact that gene order was highly conserved among different citrus species and a species from the related genus, *Poncirus*, is encouraging because it implies that information gathered in mapping projects from one segregating population of citrus may be directly related to information gathered from other genetic projects.

Genetic maps based on molecular markers have proven useful in other species for mapping genes of economic importance including monogenic traits such as disease resistance and self-incompatibility (Bournival et al. 1989; Landry et al. 1987; Sarfatti et al. 1989; Weeden 1989; Young et al. 1988) as well as polygenic (quantitative) traits such as yield and cold tolerance (Martin et al. 1989; Nienhuis et al. 1987; Paterson et al. 1988, 1990; Weeden 1989). Genetic linkage maps hold the greatest potential for increasing the efficiency of cultivar improvement programs for perennial tree fruits. Long juvenile periods and large plant size combine to hinder conventional plant breeding of fruit tree species by requiring large investments of time and land for progeny evaluation. Genetic maps of these species may provide the basis for ealy screening procedures (Tanksley et al. 1981), thus permitting breeders to make initial selections among very young progeny. Such selection would not be based on an individual's phenotype, but rather on its phenotype as predicted by its genotype at molecular marker loci known to cosegregate with a particular phenotype. Only potentially superior progeny would be grown to maturity for evaluation. By increasing the frequency of desirable phenotypes in hybrid progeny, the probability of selecting truly superior recombinants should be enhanced, and the space (as well as cost) to do so should be reduced substantially.

The populations used in this study for initiating genetic map development should also segregate for traits of economic importance. Monogenic traits that should seg-

regate include resistance to certain pests [e.g., citrus tristeza virus and citrus nematodes (*Tylenchulus semipenetrans*)] and mono versus polyembryony. Segregation is also expected for quantitative traits such as *Phytophthora* susceptibility, cold hardiness, fruit size, quality, time of maturity, salt tolerance, tree vigor, and other morphological traits. Studies to map economically important traits as well as to add additional molecular markers to the map have been initiated.

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