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Fingerprinting trifoliate orange germ plasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers

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Abstract Trifoliate orange [*Poncirus trifoliata* (L.) Raf.] is frequently used as a parent in citrus rootstock breeding, but the origin and amount of genetic diversity in germ plasm collections are poorly understood. Most accessions are self-compatible, but produce a mixture of sexual and apomictic seedlings. Variation among 48 vegetatively propagated trifoliate orange accessions was assessed at seven isozyme loci, together with the restriction fragment length polymorphisms (RFLPs) detected by 38 probe-enzyme combinations and the inter-simple sequence repeat (ISSR) markers generated by 11 primers. Isozymes and RFLPs detected few polymorphisms among accessions, although genetic analysis has shown that the common phenotype is heterozygous for four isozyme and at least four RFLP loci. ISSR amplification generated multiple banding profiles with an average of 58 fragments/primer/accession. These fragments were repeatable across DNA samples extracted from different trees of the same accession or extracted at different times, and across separate PCR runs. Seventeen unique marker phenotypes were identified. The 48 trifoliate orange accessions were classified into four major groups based on polymorphic ISSR markers. All large-flowered accessions are in group 4, while small-flowered accessions are in group 3. Many ISSR markers segregated in progeny derived by open-pollination (probably mostly selfing) of a common accession, indicating that these ISSR markers are also heterozygous. Accessions having identical genotypes for a large number of heterozygous markers are unlikely to have diverged by recombination. Thus the limited divergence we detected among most accessions most likely originated by mutation. 'Monoembryonic' and 'Simmons' differed from other accessions only in the loss of specific markers, indicating that they originated as zygotic seedlings of individuals similar to the common genotype. Three accessions recently introduced from China have relatively different fingerprints with 3–14 unique ISSR markers, and probably represent a much more divergent germ plasm that may be a valuable breeding resource.

Key words *Poncirus trifoliata* · Trifoliate orange · Isozymes · RFLPs · ISSR markers · Fingerprinting

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Introduction

The trifoliate orange [*Poncirus trifoliata* (L.) Raf.], a close relative of the genus *Citrus*, is widely used as a rootstock for citrus trees in China, Japan, and other countries in the temperate zone. Trifoliate orange is resistant to citrus tristeza virus, *Phytophthora* root rot and citrus nematode, but it is susceptible to citrus exocortis viroid and to iron chlorosis on calcareous soil (Castle 1987). Like many citrus taxa, the trifoliate orange produces polyembryonic seeds containing both sexual and apomictic embryos. About 80–90% of seedlings from open-pollinated trifoliate orange seeds develop from nucellar (apomictic) embryos that are genetically identical to their mother tree in all characters (Khan and Roose 1988). Nucellar embryony is a valuable trait for a citrus rootstock because citrus trees on zygotic rootstocks are more variable, and generally less vigorous and lower-yielding, than those on nucellar rootstocks (Roose and Traugh 1988).

In addition to its use as a rootstock, trifoliate orange is the most important source of disease resistance used by citrus breeders, and hybrids between trifoliate orange and citrus are the most commonly used rootstocks for citrus in California, Florida, and many other

regions. Therefore, an improved understanding of the genetic diversity in trifoliate orange germ plasm should allow more efficient exploitation of this material by citrus breeders.

The trifoliate orange has been grown in China for thousands of years, in Japan since at least the eighth century, and in other countries for a century (Swingle and Reece 1967). Many selections have been identified on the basis of differences in origin, growth habit, and leaf or flower morphology (Shannon et al. 1960). However, these traits are influenced to a large extent by environmental conditions. The Citrus Variety Collection at the University of California, Riverside includes 50 trifoliate orange accessions (clones) that are maintained by vegetative propagation. Many of these accession cannot be distinguished from each other by the morphology of about 12-year-old trees. Because some accessions originated from seedlings collected in nurseries or groves, they may be nucellar seedlings of other accessions. Continued maintenance of duplicate accessions in the germ plasm collection is inefficient, but it is important to correctly identify all unique germ plasms before discarding accessions.

A variety of methods can be used to measure genetic diversity. Isozyme analysis is relatively simple and inexpensive. It has been successfully used for distinguishing nucellars from zygotics of citrus (Xiang and Roose 1988) and trifoliate orange cultivars (Khan and Roose 1988), for assessing phylogenetic relationships among citrus species (Fang et al. 1994), and for identifying cultivars that have differentiated by sexual reproduction (Torres et al. 1978, 1982). However, isozyme assays generally are not useful for distinguishing cultivars derived from mutation (Roose 1988).

Restriction fragment length polymorphisms (RFLPs) are highly polymorphic in citrus (Roose 1988; Liou et al. 1996). RFLPs were used to assess genetic relationships among citrus species (Green et al. 1986) and to construct genomic maps (Durham et al. 1992; Jarrell et al. 1992; Cai et al. 1994). However, RFLPs have the disadvantages of being time consuming and labor intensive, and often involve the use of radioactive materials.

Polymerase chain reaction (PCR)-based techniques provide many additional options for cultivar fingerprinting. PCR methods using arbitrary primers have become especially popular among researchers since 1990 (Welsh and McClelland 1990; Williams et al. 1990; Caetano-Anolles et al. 1991). Of these techniques random amplified polymorphic DNA (RAPD) is the most widely applied (Tinker et al. 1993; Millan et al. 1996). In citrus, RAPD was used to identify lemon mutants (Deng et al. 1995) and chimeras (Sugawara and Oowada 1995), and to construct genomic maps (Cai et al. 1994) or to identify markers linked to important agronomic traits (Cheng and Roose 1995; Gmitter et al. 1996). However, in a preliminary experiment, four decamer primers revealed only one polymorphic fragment among 48 trifoliate orange accessions. We inferred that the efficiency of RAPD for fingerprinting trifoliate orange accessions was quite low.

Simple sequence repeats (SSRs), also called microsatellites, are tandem repeats of di-, tri-, or tetra-nucleotides which are abundant in all eukaryotic genomes (Hamada et al. 1982). SSRs have been recognized as good sources of genetic markers in many plants including *Citrus* and *Poncirus* (Akkaya et al. 1992; Wu and Tanksley 1993; Kijas et al. 1995). All five SSRs tested on two accessions of trifoliate orange were apparently heterozygous, with only a single allelic difference between the accessions (Kijas, personal communication). Standard PCR analysis of microsatellites requires a knowledge of genomic sequences flanking the SSR region to design primers that amplify the microsatellite region and reveal polymorphisms resulting from variation in repeat length. Sequencing and primer development are time consuming and expensive for a single marker.

Inter-simple sequence repeat (ISSR) amplification is a novel technique which can rapidly differentiate closely related individuals (Zietkiewicz et al. 1994). ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence such as $(CA)_8$ anchored at the 3′ or 5′ end by 2–4 arbitrary, often degenerate, nucleotides. The sequences of repeats and anchored nucleotides are randomly selected. Coupled with the separation of amplification products on a polyacrylamide gel, ISSR amplification can reveal a much larger number of fragments per primer than RAPD. ISSR has been used to investigate the genomic origins of the genus *Eleusine* (Salimath et al. 1995), to assess genetic diversity in dent and popcorn (Kantety et al. 1995) and in Douglas fir and sugi (Tsumura et al. 1996), and to identify cultivars of chrysanthemum (Wolff et al. 1995) and oilseed rape (Charters et al. 1996).

In the present paper, we report the use of isozymes, RFLPs, and ISSR markers to fingerprint trifoliate orange germ plasm accessions.

Materials and methods

Plant materials

Forty eight trifoliate orange (*Poncirus trifoliata* Raf.) accessions (Table 1) in the Citrus Variety Collection at the University of California, Riverside, California, U.S.A., and three accessions ('Rubidoux', 'Rich 16-6', and 'Flying Dragon') from the Citrus Clonal Protection Program at Lindcove, California, were sampled for isozyme analysis and DNA extraction. For 'Rubidoux', 'Pomeroy' and 'Flying Dragon', DNA was extracted at three different times to test the repeatability and reliability of ISSR markers.

DNA extraction

Total DNA was extracted from young leaves according to the protocol of Webb and Knapp (1990) with the following

Table 1 Trifoliate orange accessions characterized for isozymes, RFLPs, and ISSR markers

Name	Accession No.[a]	Flower size[b]	Origin	RFLP type[c]
Seedling	4006	–[d]	China	4
Seedling	4007	–	China	4
Seedling	4008	–	China	–
Seedling	4009	–	China	3
Argentina	3206	Large	California	4
Australian	3151	–	Australia	3
Barnes	2554	Small	Florida	3
Benecke	3338	Large	California	4
Benoit	3547	–	California	3
Christiansen	3345	Large	California	4
English dwarf	3876	Small	California	3
English large	3548	Small	California	3
Fairhope	3351	–	California	4
Flying Dragon variant	3330B	Small	Japan	3
Hiryo	3795	Small	Japan	3
Florida	2862	Large	Florida	4
Frost tetraploid	3484	–	California	3
Jacobsen	3411	Small	California	3
Kryder 5-5	3586	Large	California	4
Kryder 8-5	3218	Large	California	4
Kryder 16-6	3210	–	California	3
Kryder 55-1	3486	Large	California	4
Kryder 15-3	3217	–	California	4
Kryder 28-3	3219	Large	California	4
Kryder 55-5	3215	–	California	4
Kryder 60-2	3213	Large	California	4
Kryder medium	3212	–	California	4
Marks	3588	–	California	4
Rubidoux	838	Small	California	3
Rich 7-5	3587	Large	California	4
Rich 12-2	3209	Large	California	4
Rich 22-2	3211	Small	California	3
Taylor	3571	Small	California	3
Towne F	3572	Large	California	4
Ronnse	3570	Small	California	3
Simmons	3549	–	California	3
Texas	2861	Small	Texas	3
USDA	1498	Large	Florida	4
Webber-Fawcett #22	2552	Large	Florida	4
Yamaguchi	3412	Large	California	4
Towne G	3207	Large	California	4
Hiryu	3882	Small	Japan	3
Monoembryonic	3888	–	Japan	2
#27 China	3938	–	China	1
Nanjing	3939	–	China	3
Pomeroy	1717	Large	California	4
Flying Dragon	3330	Small	Japan	3
Rich 16-6	3485	Small	California	3

[a] Accession numbers are those used in the Citrus Variety Collection at the University of California, Riverside, California
[b] Flower sizes data mainly according to Shannon et al. (1960)
[c] RFLP type at pRLc094/*Eco*RI
[d] –, stands for no data

modification: 1.5% hexadecyltrimethylammonium bromide (CTAB) was used in both extraction and precipitation buffers. At the end of the original protocol, the DNA pellet was dissolved in 500 µl of TE. After adding 1.5 µl of RNAase (10 mg/ml), tubes were incubated at 37°C for 30 mins; 50 µl of 3 M sodium acetate and 550 µl 100% isopropanol were added to each tube to precipitate DNA at −20°C for 2 h. The DNA pellet was spun down at 12 000 rpm for 4 min, and washed with 85% and 70% ethanol once, respectively. After the ethanol evaporated, 300 µl of TE was added to suspend DNA. DNA concentration was measured with a fluorometer (Hoefer Scientific Instrument, California).

Isozyme analysis

Isozymes were analyzed according to standard protocols (Xiang and Roose 1988). The denotation of alleles and loci were according to Torres et al. (1982). A total of six enzyme systems, i.e., glutamate-oxalacetate transaminase (EC 2.6.1.1; GOT), isocitrate dehydrogenase (EC 1.1.1.42; IDH), leucine aminopeptidase (EC 3.4.11.1; LAP), malate dehydrogenase (EC 1.1.1.37; MDH), phosphoglucoisomerase (EC 5.3.1.9; PGI) and phosphoglucomutase (EC 5.4.2.2; PGM), were analyzed. One locus was scored in each system except for MDH where two loci were scored.

RFLP analysis

All 19 probes (pRLc003, 007, 015, 025, 031, 032, 038, 039, 040, 041, 049, 053, 056, 060, 066, 089, 091, 094, and 097) used in this experiment were inserts from a *Citrus jambhiri* cDNA library (Jarrell et al. 1992). Approximately 2 µg of DNA from each sample were digested with 20 units of the restriction endonucleases *Eco*RI (Promega, Wisconsin) and *Hin*dIII (Stratagene, California), respectively, according to the manufacturers' recommendations, except that 3 mM of spermidine was added. Digestion was conducted at 37°C for 14 h. DNA electrophoresis. Southern transfer, insert isolation, probe labeling, hybridization and autoradiography were according to Jarrell et al. (1992).

ISSR analysis

A total of 11 primers (see Table 2) were used to amplify DNA; these were selected after we screened 46 primers. Primers were either synthesized by Cruachem Inc. (California) or purchased from the Biotechnology Laboratory, University of British Columbia, Canada (UBC primer Kit # 9). Each 20-µl amplification reaction consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 200 µM each of dNTP, 1 µM primer, 0.01% gelatin, 2% formamide, 1 unit of *Taq* polymerase (Promega, Wiscosin), and 25 ng of template DNA. Each reaction mixture was overlaid with 50 µl of mineral oil. Amplification was performed in a 96-well Ericomp Thermal cycler (Ericomp Inc., California) under the following conditions: 4 min at 94°C for 1 cycle, followed by 30 s at 94°C, 45 s at 52°C, and 2 min at 72°C for 27 cycles, and 7 min at 72°C for a final extension. Amplification products were separated on 320 mm × 380 mm × 0.4 mm 6% non-denaturing polyacrylamide gels containing 3 M urea and 1 × TBE buffer (Zietkiewicz et al. 1994). DNA was detected by silver staining (Bassam et al. 1991).

Data analysis

Isozyme and RFLP data was not analyzed because few polymorphisms were detected among accessions. A similarity matrix using the similarity coefficient of Nei and Li (1979) was constructed for ISSR data based on the presence (coded as 1) or absence (coded as 0) of polymorphic fragments for each primer. Cluster analysis was performed with NTSYS-pc version 1.80, a numerical taxonomy and multivariate analysis software package (Rohlf 1993) using an

unweighted pair-group method, arithmetic average (UPGMA). The similarity matrix is available to readers upon request.

Results and discussion

Isozymes

All accessions had identical genotypes at the seven isozyme loci tested except as described below. Of these loci, breeding tests have shown that *Pgi-1*, *Pgm*, *Got-1*, and *Mdh-2* are heterozygous in 'Pomeroy', 'Rubidoux', and 'Flying Dragon' (Khan and Roose 1988). At *Pgi-1*, 'Seedling 4008' and '#27 China' were *GS*, 'Simmons' and 'Monoembryonic' were *SS*, and all the others were *FS*. At *Pgm*, 'Frost tetraploid' was *FF*, but all the others were *FS*. These results show that 'Seedling 4008' and '#27 China' are unique accessions because they have a unique allele, *Pgi-1G*. 'Simmons', 'Monoembryonic' and 'Frost tetraploid' have genotypes consistent with an origin as zygotic seedlings of common accessions. Other studies (Torres et al. 1978, 1982; Hirai et al. 1986; Fang et al. 1994) also observed little isozymic variation among trifoliate orange accessions. Isozymes are of limited value for fingerprinting these trifoliate orange accessions.

RFLPs

A total of 38 probe-enzyme combinations (PECs) generated 80 fragments. However, 17 PECs had only one monomorphic fragment. Of these 80 fragments, ten fragments from five different PECs were polymorphic. Although a genetic interpretation of all RFLP patterns is not possible without segregation analysis, at least four RFLP loci must be heterozygous because different *Citrus* × *Poncirus* hybrids inherit different restriction fragments from their *Poncirus* parent (data not shown). The most polymorphic PEC was pRLc094/*Eco*RI which separated these accessions into four phenotypes (Table 1). Large-flowered accessions have a type-4 pattern, while small-flowered accessions have type 3. Types 1 and 2 each have only one accession, '#27 China' and 'Monoembryonic', respectively. '#27 China' had unique RFLP fragments with two PECs, pRLc060/*Hin*dIII and pRLc094/*Eco*RI. Two PECs, i.e., pRLc031/*Eco*RI and pRLc053/*Eco*RI, could differentiate 'Monoembryonic' from all other accessions, but these involved only loss of fragments from the basic pattern. Three phenotypes were observed with pRLc053/*Hin*dIII: '#27 China', 'Monoembryonic' and 'Simmons', and all other accessions. Although the pRLc089/*Eco*RI displayed certain polymorphisms, it was hard to score them reliably. Overall, only '#27 China' had unique RFLP fragments. 'Monoembryonic' and 'Simmons' involved loss of fragments from the basic pattern, as expected in zygotic seedlings resulting from selfing or crossing between identical genotypes. Komatsu et al. (1993) observed little RFLP variation among 16 trifoliate orange accessions when M13 and the rRNA gene of rice were used as probes. The RFLP data suggest that the trifoliate orange germ plasm in our collection has relatively low genetic diversity despite being heterozygous at four isozyme and at least four RFLP loci. Of these eight loci, seven have been mapped (Jarrell et al. 1992; Cai et al. 1994) and only one pair of RFLPs show linkage, so heterozygosity appears to be distributed over many chromosomal regions.

ISSR markers

ISSR amplification from all DNA samples resulted in multiple banding profiles for all 11 primers. The amplified fragment sizes ranged from 80 bp to 3 kb with the scorable region being from 150 bp to 2.5 kb (Fig. 1). The number of fragments per primer ranged from 36 [BDB$(TCC)_5$] to 91 [$(AG)_8$YT] with an average of 58 (Table 2). Of the total 637 scorable fragments, 65 were polymorphic among the accessions (Figs. 1 and 2). Among these 11 primers, three revealed little polymorphism (two polymorphic fragments per primer), while HVH$(CA)_7$T generated the most (15 polymorphic fragments).

Reliability and repeatability are essential for a technique to be used in fingerprinting. Though RAPD is widely used in biology, problems of reliability and repeatability have been noted (Ellsworth et al. 1993). Many factors may affect amplification, such as the concentration of the Mg^{2+}, thermal cycler used and even the source of polymerase. In the current experiment, the amplification of ISSR markers was consistent across DNA samples from the same accession in different locations (Riverside vs Lindcove), or else extracted from different leaves of the same tree, and across separate PCR runs, with over 99% of the scoreable fragments reproducible. Only very faint fragments were not reproducible and such fragments were not scored in this study. We obtained identical patterns with samples amplified on thermocyclers from different manufacturers. The high reproducibility of ISSR markers may be due to the use of longer primers and higher annealing temperatures than those used for RAPD. We have not evaluated reproducibility between laboratories.

To select primers for the present study, we tested 46 ISSR primers using trifoliate orange and citrus templates. These primers had either different core repeats or anchored nucleotides. Twenty two of the 46 primers generated clear multiplex profiles (Fang and Roose, manuscript in preparation). However, the other 24 produced either no products at all or else smears that could not be scored. Modification of PCR amplification conditions and staining did not improve the patterns much. We believe that the poor results with these 24 primers were due to characteristics of the primers or to the

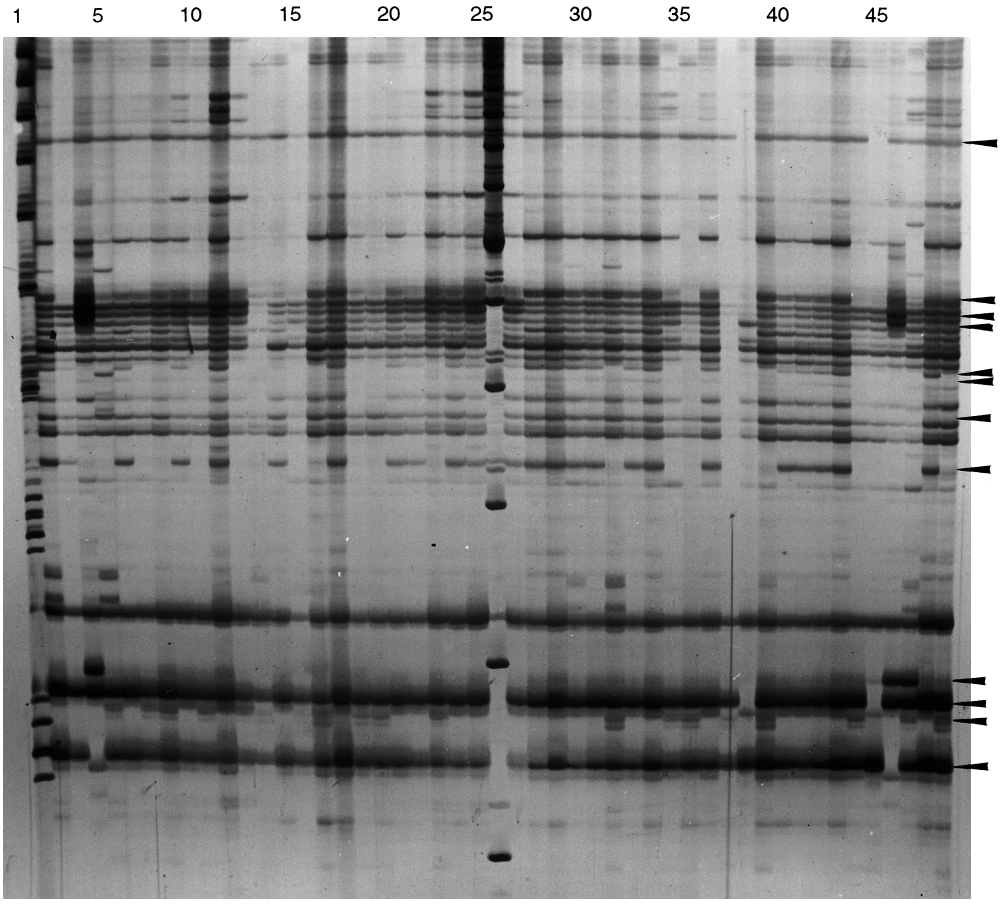

Fig. 1 ISSR profiles amplified from DNA of trifoliate orange accessions using primer $HVH(CA)_7T$. The order of lanes is the same as the order of accessions in Table 1 except that *lanes 1 and 25* are 123-bp (the smallest band is 246 bp) and 100-bp ladder markers (the smallest band is 200 bp), respectively. *Arrows* indicate 12 of the 15 polymorphic fragments scored

relative abundance of priming sites in trifoliate orange and citrus DNA. According to Wang et al. (1994), in plant nuclear DNA the dinucleotides sequence $(AT)_n$ is the most abundant, followed by $(A)_n/(T)_n$ and $(AG)_n/(CT)_n$. However, in our experiment, no products were amplified when $(AT)_8RT$, $(AT)_8RG$ or $(AT)_8RC$ was used. Similarly, no amplification products were detected with primers containing $(TA)_8$ repeats, regardless of the anchored nucleotides. Primers based on $(CA)_n$ and $(TCC)_n$ gave excellent fingerprint patterns, suggesting that these repeats are abundant in *Citrus* and *Poncirus*.

Zietkiewicz et al. (1994) noted that primers anchored at the 5′ end displayed broader specificity than those anchored at the 3′ end. We found that in *Poncirus* and *Citrus*, 5′-anchored primers generally produced clearer patterns but fewer and larger fragments than 3′-anchored primers. As a general rule, the higher the density of repeats in a genome, the more specific the primers and the more stringent the PCR conditions that should be used to limit the number of amplified products and to optimize their resolution on a gel.

Table 2 ISSR primers used in this experiment and polymorphic fragments obtained

Primers[a]	Fragments	Polymorphic fragments
$HVH(CA)_7T$	71	15
$HVH(TG)_7T$	51	4
$(GA)_8YG$	57	7
$HVH(TCC)_5$	42	2
$BDB(CA)_7C$	54	10
$BDB(TCC)_5$	36	2
$(TCC)_5RY$	48	7
$(AG)_8YT$	91	3
$VHVG(TG)_7$	60	2
$DBDA(CA)_7$	52	8
$(AG)_8YC$	75	5
Total	637	65

[a] R = purine, Y = pyrimidine, B = non-A, D = non-C, H = non-G, V = non-T

Although using radioactively labeled primers (Zietkiewicz et al. 1994) or dCTP (Kantety et al. 1995) in PCR amplification for ISSR achieved good results (sharp bands on a film), these methods are expensive [for example, Kantety et al. (1995) used 1 µl of

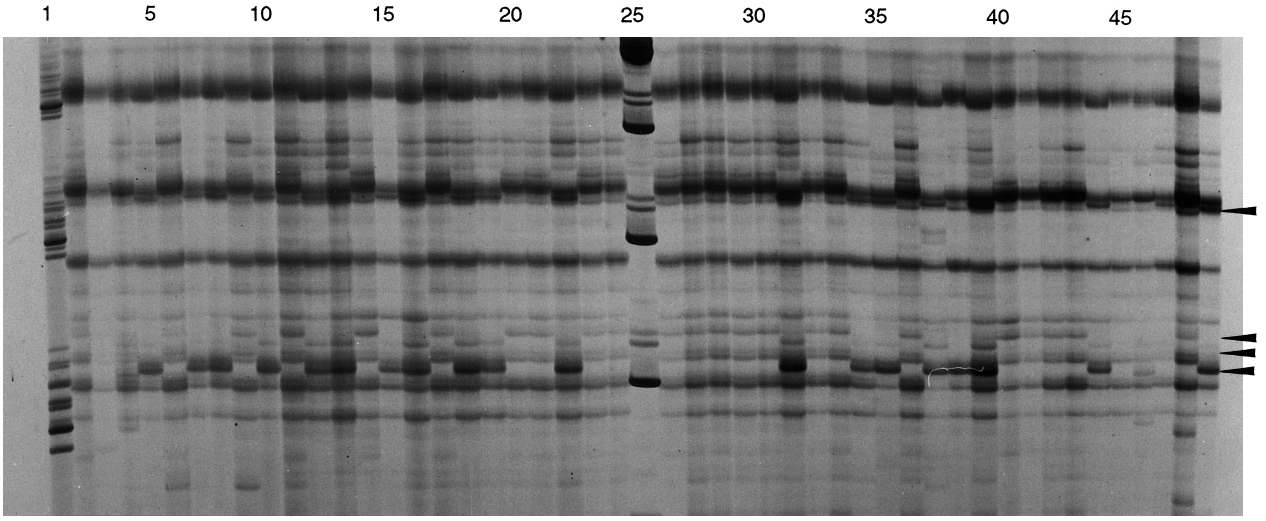

Fig. 2 ISSR profiles amplified from DNA of trifoliate orange accessions using primer $(GA)_8YG$. The order of lanes is the same as the order of accessions in Table 1 except that *lanes 1 and 25* are 123-bp (the smallest band is 369 bp) and 100-bp ladder markers (the smallest band is 400 bp), respectively. *Arrows* indicate four of the seven polymorphic fragments scored

3000 Ci/mmol ^{32}P-dCTP in one reaction] and can result in evaporation of radioactive materials during amplification (Trentmann et al. 1995). In preliminary experiments, we compared fingerprints detected by ^{32}P-dCTP labeling and radioautography with silver staining. Our results (data not shown) indicated that silver staining detected all of the products detected by autoradiography. Silver staining is much cheaper and faster (usually within 2 h after electrophoresis). We noted that it is important to use high-quality chemical regents during the silver staining process.

Fingerprinting accessions

Of the 80 RFLP and 637 ISSR fragments, only ten and 65 were polymorphic, respectively. With either technique, about 10% of the markers were polymorphic. Another measure of the power of these techniques is the number of unique (accession-specific) markers detected. One unique isozyme allele, three unique RFLP fragments, and 17 unique ISSR fragments were detected in this study. Our results indicate that genetic diversity within the trifoliate orange is quite low. *P. trifoliata* is a distinct species in the subtribe *Citrinae* which consists of *Poncirus*, *Citrus*, and four other genera (Swingle and Reece 1967). *P. trifoliata* originated in the temperate zone in China, while all other species in this subtribe originated in a tropical or subtropical region. Although *P. trifoliata* can freely hybridize with the other genera, geological isolation has greatly reduced the opportunities for gene exchange between *P. trifoliata* and the other species. Moreover, even if *P. trifoliata* grows together with *Citrus* trees, *P. trifoliata* flowers about 20–30 days earlier than *Citrus*. Because of this, natural hybridization between *P. trifoliata* and *Citrus* rarely occurs (Khan and Roose 1988). The polymorphic markers detected in the present study are almost all unique to *P. trifoliata*; therefore the diversity we have observed is unlikely to be caused by hybridization with *Citrus*. One factor contributing to the extremely low diversity among the accessions in our collection may be their narrow genetic base. Trifoliate orange was first imported into the USA from Japan in 1869 (Hodgson 1967). Most of the modern accessions originated from these old lines by the selection of mutations or by self-pollination.

Based on 65 polymorphic ISSR fragments, a similarity matrix was generated using the coefficient of Nei and Li (1979). Figure 3 is the dendrogram constructed by UPGMA cluster analysis. Based on this dendrogram, all accessions can be separated into four major groups.

Group 1 consists of '#27 China' and 'Seedling 4008'; any one of 11 ISSR primers tested can discriminate them from the other accessions. Moreover, 14 of 65 polymorphic ISSR fragments are unique to these two accessions. In addition to the ISSR markers, these two accessions are distinct from the other accessions in having the unique allozyme allele *Pgi-1G*. '#27 China' had unique RFLP profiles at three PECs ('Seedling 4008' was not tested for RFLPs). They were introduced as seeds from China in the 1980s. No marker differences were found between these two accessions, which may indicate that they are nucellar seedlings from the same tree or accession in China. The genetic uniqueness of group 1 indicates that, although our collection includes relatively little genetic diversity, more variation exists in China. Recent discovery of a new evergreen *Poncirus* species, *P. polyandra* (Ding et al. 1984), is further evidence that more diversity exists in China.

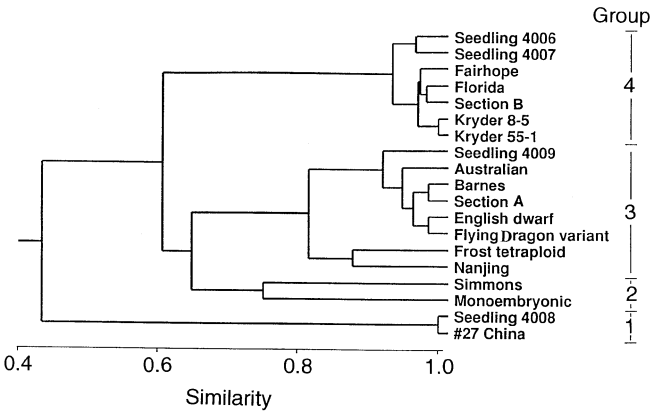

Fig. 3 Dendrogram illustrating the genetic relationships among 48 trifoliate orange accessions. The dendrogram was drawn based on UPGMA cluster analysis, using the similarity matrix derived from 65 polymorphic ISSR fragments. Section A in group 3 includes 'Benoit', 'English large', 'Flying Dragon', 'Hiryo', 'Hiryu', 'Jacobsen', 'Kryder 16-6', 'Rich 16-6', 'Rich 22-2', 'Ronnse', 'Rubidoux', 'Taylor' and 'Texas'. Section B in group 4 includes 'Argentina', 'Benecke', 'Christiansen', 'Kryder 5-5', , 'Kryder 15-3', 'Kryder 28-3', 'Kryder 55-5', 'Kryder 60-2', 'Kryder medium'. 'Marks', 'Pomeroy', 'Rich 7-5', 'Rich 12-2', 'Towne F', 'Towne G', 'USDA', Webber-Fawcett #22' and 'Yamaguchi'

Group 2 is composed of 'Monoembryonic' and 'Simmons'. Though they have different profiles from the other groups for PGI isozymes, several RFLP PECs and ISSRs, they had no unique alleles or fragments. Because 'Monoembryonic' differs from the accessions in group 3 only in lacking six restriction fragments and 21 ISSR markers, 'Monoembryonic' might have originated from a zygotic embryo of a genotype in group 3. This origin is consistent with the genetic control of the polyembryonic trait by one or two genes with polyembryony being dominant to monoembryony (Cameron and Soost 1979). 'Simmons' apparently arose in a similar fashion because it lacks three restriction fragments and 13 ISSR markers found in the basic pattern of group 3. These two accessions can be differentiated from each other by PGI isozymes, RFLPs or ISSR markers.

Group 3 includes 20 accessions based on ISSR markers. All group 3 accessions have a type-3 pattern for pRLc094/*Eco*RI. Unfortunately, we do not have complete information about their flower sizes. However, our available data show that all small-flowered accessions are in this group. Moreover, within this group, 'Nanjing', 'Frost tetraploid', 'English dwarf', 'Flying Dragon variant', 'Seedling 4009', 'Australian', and 'Barnes' could be distinguished from the others by the presence or absence of ISSR fragments. 'Nanjing', which was introduced from China in the 1980s, is the most distinct. It has three unique ISSR fragments, indicating that it is unlikely to be a self-pollinated seedling of a common accession, and that it may represent a unique germ plasm. The other 13 accessions in this group were identical for all markers scored. Some of these may be the same cultivar but under different names. For example, 'Flying Dragon' is called 'Hiryo' (Swingle and Reece 1967) or 'Hiryu' (Komatsu et al. 1993) in Japan. Moreover, all of these three accessions came from Japan. Others may differ in mutations that affect horticultural performance. 'Flying Dragon', 'Hiryo' and 'Hiryu' have curved shoots and thorns which are morphologically different from the other accessions, and 'Flying Dragon' acts as a strongly dwarfing rootstock for citrus. The evidence presented here suggests that these characters arose by mutations in this accession. Such observations also point out that characterization of molecular markers alone does not identify all useful germ plasm.

Group 4 is composed of 24 accessions. All of them have the type-4 pattern for RLc094/*Eco*RI. Our available data show that all large-flowered accessions are in this group. There are few differences among accessions within this group. 'Seedling 4006', 'Seedling 4007', 'Fairhope', and 'Florida' have unique fingerprints. 'Kryder 8-5' and 'Kryder 55-1' had the same banding profiles which are different from those of other accessions. The other 18 accessions have identical fingerprints. The nine 'Kryder' accessions were collected from the same grove in Claremont, California. It is very likely that the six 'Kryder' accessions with identical fingerprints originated from nucellar embryos of the same source. Similarly, 'Towne F' and 'Towne G' probably originated as nucellar seedlings from the same source tree.

Studies of the inheritance and linkage relationships of ISSR markers are in progress. For the markers studied here, we determined the segregation patterns of markers amplified with two primers, $HVH(TG)_7T$ and $(TCC)_5RY$, in 21 zygotic progeny derived by open-pollination of 'Flying Dragon' (Cheng and Roose, 1995). With each primer, eight markers segregated in ratios not significantly different from the 3 : 1 ratio expected from selfing. One additional marker showed skewed segregation, with an excess of the no-product class. These results, and studies in progress in other larger populations, indicate that most ISSR fragments segregate as dominant markers. The proportion of ISSR markers showing skewed segregation is similar to that observed for isozymes, RFLPs, and RAPDs in the same cross (Roose, unpublished data).

In conclusion, ISSR markers are the most powerful of these three techniques for fingerprinting closely related accessions such as those of trifoliate orange. Although most ISSR alleles are dominant, rather than co-dominant, ISSR amplification of trifoliate orange DNA offers several advantages over isozymes, RFLPs, and even RAPDs; the major one being rapid production of a large number of markers in a cost-effective manner. ISSR amplification has great potential in plant breeding and germ plasm evaluation, especially for

fingerprinting narrow-based germ plasms like those present in trifoliate orange.

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