DNA Amplified Fingerprinting, A Useful Tool for Determination of Genetic Origin and Diversity Analysis in Citrus

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Additional index words. PCR, polymorphism, plant breeding, RAPD

Abstract. We used three short repetitive nucleotide sequences [(GTG)5, (TCC)5, and (GACA)4] either as radiolabeled probes for hybridization with restricted citrus DNA or as single primers in polymerase chain reaction amplification experiments with total genomic DNA. We tested the ability of the sequences to discriminate between seedlings of zygotic or nucellar origin in the progeny of a Volkamer lemon (Citrus volkameriana Ten. & Pasq.) tree. The genetic variability within two species [Citrus sinensis (L.) Osbeck (sweet oranges) and Citrus reticulata Blanco and relatives (mandarins)] also was evaluated. DNA amplified fingerprinting with single primers was the more successful technique for discriminating between nucellar and zygotic seedlings. Although we were not able to distinguish among 10 cultivars of C. sinensis, all 10 C. reticulata cultivars tested were distinguishable. However, it still is difficult to identify the putative parents of a hybrid plant when the two parental genomes are closely related.

To our knowledge, repetitive sequences, called minisatellites or variable number of tandem repeats (VNTR), have been found in the genomes of all eukaryotes tested (Epplen, 1988; Roeper et al., 1990; Tautz and Renz, 1984; Weising and Kahl, 1990). Used as probes, these sequences may yield DNA fingerprints, which are useful for genetic analysis and individual identification (Matsuyama et al., 1992; Nybom et al., 1990; Ryskov et al., 1988; Vassart et al., 1987). Expensive and time-consuming techniques, such as labeling with radioactive probes and purification of large quantities of DNA, may be replaced by using fragments generated by random primers in a polymerase chain reaction (PCR) as markers (Welsh and Mc Clelland, 1990; Williams et al., 1990). For rapid identification of individual, repetitive sequences scattered throughout the genome seem more appropriate because the probability of revealing some polymorphism likely will be greater. PCR methods based on families of repeated sequences have been tested successfully [e.g., Alu-directed PCR in human genome analysis (Nelson et al., 1989)].

We reported on the occurrence of several minisatellite sequences in citrus genomes by Southern blot hybridization. These sequences were used as primers for PCR amplification. The efficiency of this technique for distinguishing zygotic from nucellar (apomitic) seedlings was evaluated. The phenomenon of facultative apomixis via nucellar embryony, a genetic trait common to many citrus cultivars (Frost and Soost, 1968), is a hindrance in breeding programs already hampered by long generation time, large space requirements by individuals, and little knowledge of inheritance of important traits. Many important citrus cultivars produce a polylembryonic mixture of nucellar and recombinant sexual embryos, and it may be necessary to distinguish the two types of seedlings as early as possible. For example, nucellar seedlings are used for roostock propagation because they are identical to the mother plant, but recombinant zygotic seedlings are of interest in breeding programs. These two types of seedlings are difficult to identify based solely on their morphological characters. The only accurate discriminant morphological character is the trifoliate leaf morphology, seen when Poncirus trifoliata (L.) Raf. is the paternal parent of zygotic seedlings. However, using isozyme markers brought a major improvement in the distinction of the two types of seedlings in several citrus progenies (Soost and Williams, 1980; Torres et al., 1982). We also evaluated the VNTR-PCR technique for its ability to distinguish between sweet orange cultivars and to reveal polymorphism among mandarin cultivars.

Table 1. List of Citrus trees studied.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Botanical name</th>
<th>Accession no.</th>
<th>Mode of propagation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet oranges</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 42</td>
<td>Budding</td>
</tr>
<tr>
<td>Pineapple</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 573</td>
<td>Shoot-tip grafting</td>
</tr>
<tr>
<td>Tarocco</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 43</td>
<td>Budding</td>
</tr>
<tr>
<td>Parson Brown</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 299</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Shaminoti</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 354</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Doublefine</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 217</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Whashington navel</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 8</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Valencia late</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 352</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Sanguinelli</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 232</td>
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</tr>
<tr>
<td>Cadenera</td>
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<td>SRA 251</td>
<td>Somatic embryo</td>
</tr>
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<td>Hamlin</td>
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</tr>
<tr>
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<td>SRA 274</td>
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<td>Willowleaf</td>
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<td>SRA 339</td>
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</tr>
<tr>
<td>King of Siam</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 594</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Clementina Nules</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 585</td>
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</tr>
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<td>Dancy</td>
<td>C. sinensis (L.) Osb.</td>
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<td>SRA 145</td>
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</tr>
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<td>B6X19</td>
<td>Somatic embryo</td>
</tr>
<tr>
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<td>C. sinensis (L.) Osb.</td>
<td>SRA 112</td>
<td>Somatic embryo</td>
</tr>
<tr>
<td>Temple</td>
<td>C. sinensis (L.) Osb.</td>
<td>SRA 348</td>
<td>Shoot-tip grafting</td>
</tr>
</tbody>
</table>

Plant material. Samples from 19 seedlings of Citrus volkameriana (numbers 2 to 20) and their mother (number 1), as well as 10 sweet orange [C. sinensis (L.) Osbeck] and 10 mandarin cultivars were harvested from the field germplasm collection of Centre de Coopération

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were washed in 1
peratures were the same as those for the an-
hybridization temperature (specific to each primer), and 1
sequence as primers) were evaluated for
and VNTR-directed PCR (PCR with repet-
ly cloned genomic DNA from (Maniatis et al., 1982). The hybridization tem-
G-50 resin (Pharmacia, Uppsala, Sweden)
nonincorporated nucleotides were removed
T4 (New England Biolabs, Beverly, Mass.);
with polynucleotide kinase of bacteriophage
synthetic oligonucleotides were end-labeled
0.5% skim milk in substitution for salmon
Denhart’s solution, 10% dextran sulfate, and
buffer (0.04 M tris-acetate; 0.001 M EDTA).
sequently staining with ethidium bromide and
agarose or 8% polyacrylamide gels, subse-
ucts were analyzed by electrophoresis on 1.5%
(GACA)4 and (TCC)5. Amplification prod-
annaling temperature (specific to each primer), and 1
min at 72C was allowed to complete the syn-
cycler (model 240 VAC; Perkin-Elmer Cetus,
reaction was performed in a DNA thermal
sample DNA (5 to 20 ng). The mixture was
graphed at –80C with intensifying screens.
Digested DNA samples (2.5
manufacturer’s recommendations (Gibco-
nucleases
samples were digested with restriction endo-
CcAAGACAGACAAGACA-3’, (TCC)5: 5’-
GACAGACAGACAGACA-3’, (TCC)5: 5’-
Biosystems, Foster City, Calif.): (GTG)5: 5’-
GACACAGACACAGACA-3’, (TCC)5: 5’-
GACGGTGGGTGGTGGTG-3’, (GACA)4: 5’-
TAACGTGGGTGGTGGTG-3’, (GACA)4 and (TCC)5. Amplification prod-
ultraviolet light.
DNA hybridization procedure. Citrus DNA
samples were digested with restriction endo-
nucleases HinII and DraI according to the manu-
frequencies or absence of amplified fragments in
DNA amplified fingerprinting (DAF) of mandarins was scored as 1 (present) or 0 (absent).
The pairwise similarities between all indi-
vidual genotypes were calculated using Nei’s
estimator (Nei and Li, 1979): S = 2nxy /(n x  + n y ),
in which n x  and n y  are the number of fragments
in genotypes x and y, respectively, and n xy  is
the number of fragments shared by the two
genotypes.

Results

Identifying zygotic and nucellar seedlings. Hybridization with labeled oligonucleotides
and VNTR-directed PCR (PCR with repet-
tive sequences as primers) were evaluated for
their ability to discriminate between nucellar
or zygotic seedlings of C. volkameriana. The
results were compared to those obtained from
isozyme analysis using four heterozygous loci
(Ollitrault et al., 1994), which showed that C.
volkameriana individuals 2 to 9 and 13 were
nucellar seedlings and individuals 10 to 12 and
14 to 20 were seedlings of zygotic origin.
We have used the minisatellites [(GTG)5, (TCC)5, and (GACA)4] as oligonucleotide
probes for Southern hybridizations with re-
stricted DNA from several C. volkameriana
seedlings. The resulting autoradiography re-
vealed restriction fragment length polymor-
phism (RFLP), but the high background level
and the poor resolution of the bands did not
make it possible to use this technique for
routine analysis (data not shown). Neverthe-
less, this experiment showed the presence of
numerous minisatellite sequences in C.
volkameriana and, by extension, in citrus ge-
nomes.
When the (GTG)5 sequence was used as
primer in the amplification reaction, the re-
sulting DAFs for individuals 2 to 9 and 13
were similar to the mother plant (Fig. 1A). The
other plants were characterized by the loss of
some bands or the presence of new amplified
fragments when compared to the mother DAF

Fig. 1. Polymerase chain reaction amplification of genomic DNA from C. volkameriana seedlings with
primers (A) (GTG)5 and (B) (TCC)5. Fragments were separated on (A) 1.5% agarose or (B) 8%
polyacrylamide gel. The upper numbers correspond to the individual designations of C. volkameriana
seedlings. M = molecular weight markers (in base pairs).

Identification of genetic diversity. The pres-
(individual 1). These results fully agree with those obtained with isozymes. However, such clear-cut results were not obtained with primers (TCC)5 (Fig. 1B) and (GACA)4 (data not shown). With (TCC)5, three zygotic individuals (17, 18, and 20) had DAFs identical to those of nucellar seedlings, but with (GACA)4 primer, one zygotic sample (20) had a profile similar to that of the mother (data not shown).

**Genetic diversity estimation.** Using the same VNTR primers, we evaluated the genetic diversity within two groups of citrus plants: sweet oranges (Fig. 2), mandarins, and mandarin relatives (Fig. 3). With these primers, it was not possible to find a single difference among the DAFs of the 10 C. sinensis cultivars.

With the mandarin cultivars, combinations of fingerprints obtained with each of the three primers made it possible to distinguish each cultivar from the others (Fig. 3). To quantify the diversity, we constructed a similarity matrix based on 25 polymorphic amplified fragments (Table 2). This similarity is linked to the probability that one amplified fragment from one sample also will be found in another (Nei and Li, 1979).

According to genotype similarities, the mandarin cultivars may be grouped into several clusters. ‘Dancy’ and ‘Ponkan’ mandarins (C. reticulata) are closely related (0.88 of similarity). ‘Malvasio’ mandarin (C. reticulata) seems more distantly related to these two clones. Another cluster is composed of ‘Cleopatra’ mandarin (C. reshni), and ‘Willowleaf’ mandarin (i.e., with the mandarins known to be less heterozygous). The VNTR-directed PCR technique is simple and rapid and avoids using radioactivity. The DAFs obtained with each primer were highly reproducible. Different DNA preparations from the same individual gave identical results (data not shown). This reproducibility was obvious in the case of DAFs obtained for 10 clones of C. sinensis because no variation could be detected among these cultivars after amplification with (GACA)4 or (GTG)5 (Fig. 2).

**Identifying nucellar and zygotic seedlings.** Successfully using molecular markers to distinguish zygotic from nucellar seedlings depends on the number of loci detected and the degree of plant heterozygosity. Fingerprints with minisatellites as probes indicated the presence of numerous copies of three minisatellites, and consequently, we used these minisatellites as primers for PCR amplification of citrus genomic DNA.

The DAFs obtained with each primer for all the nucellar seedlings were identical. The primers differed in their ability to discriminate between the two types of seedlings and this difference correlated with the number of amplified fragments. The DAFs obtained with (GTG)5 had the most bands and were consequently the most discriminative. Primer (TCC)5 produced fewer bands and was less useful. However, primers giving the most fragments also produced a higher background and pictures of lower quality (Fig. 1A compared to Fig. 1B). Nevertheless, the relatively high level of background does not prevent the reproducibility of the results.

With a single primer (GTG)5 and 5 ng of DNA extracted by a fast procedure, it was possible to distinguish between nucellar and zygotic seedlings; four isozyme systems (aspartate aminotransferase, phosphoglucone isomerase, malate dehydrogenase, and isocitrate dehydrogenase) were necessary to accomplish the same task (Ollitrault et al., 1994). The level of efficiency likely will vary with progeny from other citrus species.

Zygotic seedlings may arise by outcrossing or self-fertilization. The individuals resulting from outcrossing were easy to detect because their DAFs were characterized by additional bands compared to nucellar patterns; this was the case of individual 15 with primer (TCC)5 (Fig. 1), where four additional bands were revealed. In contrast, zygotic plants resulting from self-fertilization may have DAFs characterized by the absence of some bands compared to the nucellar pattern. This was the case for individual 11 with primer (TCC)5 (Fig. 1); this distinction was relatively easy with C. volkameriana because its genome is highly heterozygous. Most citrus clones are heterozygous at many loci, and analysis of many heterozygous loci can distinguish, with high probability, zygotic seedlings produced by self-pollination from nucellar seedlings (Roose, 1988). The VNTR-PCR technique should be tested in less favorable conditions (i.e., with the mandarins known to be less heterozygous).

**Gene diversity estimation.** As demonstrated by isozyme analysis (Ollitrault and Faure, 1992; Torres et al., 1978), the intraspecific diversification processes are fundamentally different for C. sinensis and C. reticulata and relatives. Sweet orange cultivars arose by successive mutations from an ancestral apomictic-
Table 2. Similarity matrix of mandarin cultivar genotypes.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Cleopatra</td>
<td>1.00</td>
<td>0.91</td>
<td>0.90</td>
<td>0.82</td>
<td>0.44</td>
<td>0.82</td>
<td>0.73</td>
<td>0.59</td>
<td>0.67</td>
</tr>
<tr>
<td>2 Nules</td>
<td>0.91</td>
<td>1.00</td>
<td>0.90</td>
<td>0.76</td>
<td>0.60</td>
<td>0.74</td>
<td>0.86</td>
<td>0.63</td>
<td>0.78</td>
</tr>
<tr>
<td>3 Willowleaf</td>
<td>0.90</td>
<td>0.90</td>
<td>1.00</td>
<td>0.63</td>
<td>0.44</td>
<td>0.71</td>
<td>0.86</td>
<td>0.63</td>
<td>0.74</td>
</tr>
<tr>
<td>4 Temple</td>
<td>0.82</td>
<td>0.76</td>
<td>0.63</td>
<td>1.00</td>
<td>0.59</td>
<td>0.63</td>
<td>0.74</td>
<td>0.53</td>
<td>0.67</td>
</tr>
<tr>
<td>5 King of Siam</td>
<td>0.44</td>
<td>0.60</td>
<td>0.44</td>
<td>0.59</td>
<td>1.00</td>
<td>0.53</td>
<td>0.78</td>
<td>0.53</td>
<td>0.67</td>
</tr>
<tr>
<td>6 Dancy</td>
<td>0.82</td>
<td>0.74</td>
<td>0.71</td>
<td>0.63</td>
<td>0.53</td>
<td>1.00</td>
<td>0.74</td>
<td>0.57</td>
<td>0.53</td>
</tr>
<tr>
<td>7 Ponkan</td>
<td>0.73</td>
<td>0.86</td>
<td>0.74</td>
<td>0.78</td>
<td>0.59</td>
<td>0.74</td>
<td>1.00</td>
<td>0.53</td>
<td>0.78</td>
</tr>
<tr>
<td>8 Malvasio</td>
<td>0.59</td>
<td>0.63</td>
<td>0.47</td>
<td>0.63</td>
<td>0.53</td>
<td>0.57</td>
<td>0.63</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td>9 Wilking</td>
<td>0.53</td>
<td>0.67</td>
<td>0.53</td>
<td>0.67</td>
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<td>0.38</td>
<td>0.44</td>
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<tr>
<td>10 Satsuma</td>
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<td>0.78</td>
<td>0.67</td>
<td>0.70</td>
<td>0.74</td>
<td>0.67</td>
<td>0.70</td>
<td>0.78</td>
<td>0.70</td>
</tr>
</tbody>
</table>

In contrast, a great diversity in DAFs was revealed in the mandarin group. The similarities observed were, in general, in good agreement with previous studies. The fragments amplified with short primers, such as RAPD markers, probably are dominant (Williams et al., 1990). This situation also may exist with minisatellites used as primers. With the same primer, the number of amplified fragments likely will vary from one individual to the other, according to the heterozygosity at the corresponding loci. When more loci are detected in a particular genome rather than in another, the similarity values may shift because fragments corresponding to homozygous loci will not be recorded as two fragments. This shift would explain the lower degree of similarity between ‘Wilking’ mandarin (C. nobilis × C. deliciosa) and ‘Willowleaf’ mandarin (C. deliciosa) than that observed between ‘Wilking’ and ‘King of Siam’ (C. nobilis) mandarins, although theoretically, ‘Wilking’ mandarin should be at equal distance from ‘Willowleaf’ and ‘King of Siam’ mandarins. Such a bias likely will be limited with the use of additional primers or by codominant marker analysis (e.g., RFLP or isozymes).

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<tr>
<td>3 Willowleaf</td>
<td>0.90</td>
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<tr>
<td>6 Dancy</td>
<td>0.82</td>
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</tr>
</tbody>
</table>

The similar patterns observed with sweet oranges and mandarins agree with the close phylogenetic relationships of these species (Barrett and Rhodes, 1976; Scora, 1975). Consequently, it is difficult to determine whether ‘Temple’ mandarin is of interspecific origin because of the low level of polymorphism between sweet orange and mandarin. Using additional primers or RFLP may provide more information.

The presence in citrus genomes of three distinct, single-repeat, DNA sequences was demonstrated. Using minisatellites as primers in PCR of genomic DNA is technically easy and rapid and avoids constraints due to limited plant material. Although their use as probes in a classical technique of RFLP fingerprinting is potentially more powerful in detecting polymorphism, the resulting hybridization profiles are more difficult to analyze than electrophoretic profiles of amplified fragments.

The DAFs observed after VNTR–PCR techniques make it possible to distinguish nuclear and zygotic seedlings and to distinguish each of the analyzed mandarins. In contrast, the 10 orange cultivars studied had identical profiles. These results were similar to those obtained with isozymes or RFLPs. Thus, VNTR–PCR techniques can be used for various analyses in citrus breeding, rootstock propagation, and genomic mapping.

Literature Cited


