Theor Appl Genet (1997) 95 : 408–417

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Identification of closely related citrus cultivars with inter-simple sequence repeat markers

Received: 9 September 1996 / Accepted: 4 April 1997

Abstract Inter-simple sequence repeat (ISSR) markers generated by 22 primers were tested for their ability to distinguish among samples from 94 trees of 68 citrus cultivars. Within each of the six cultivar groups studied, most of these cultivars are so closely related that they are difficult to distinguish by other molecular-marker techniques. ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite sequence anchored at the 3′ or 5′ end by 2–4 arbitrary, often degenerate, nucleotides. The amplification products were separated on non-denaturing polyacrylamide gels and detected by silver staining. ISSR banding profiles were very repeatable on duplicate samples. Different citrus species had very different fingerprint patterns. Within *Citrus sinensis* (L.) Osbeck and *C. paradisi* Macf., in which all cultivars have originated by the selection of mutants, ISSR markers distinguished 14 of 33 sweet orange and 1 of 7 grapefruit cultivars. Five of six lemon cultivars were discriminated by ISSR markers. Many differences were found among mandarin cultivars; however, all five satsuma cultivars analyzed had identical ISSR fingerprints. Four of five citrange cultivars were distinguishable, but 'Troyer' and 'Carrizo' had identical ISSR fingerprints. 'Kuharske Carrizo' citrange, which has better citrus nematode resistance than other 'Carrizo' citrange accessions, had unique ISSR fingerprints. Three ISSR markers that differentiated certain sweet orange cultivars were hybridized to Southern blots of sweet orange DNA digested with different restriction endonucleases. The sweet orange cultivars tested could be distinguished by these ISSR-derived RFLP markers. Moreover, one ISSR marker unique to 'Ruby' blood orange was observed in its progeny trees.

Communicated by G. E. Hart

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Key words *Citrus* · *Poncirus* · ISSR markers · RFLPs · Cultivar identification

Introduction

Many citrus cultivars are very closely related, apparently having diverged by mutations that alter specific horticultural traits. These mutations can be maintained because citrus is usually propagated vegetatively by grafting the scion cultivar onto a rootstock. In addition, many citrus cultivars produce apomictic seedlings through nucellar embryony, and nucellar seedlings that differ in horticultural traits or lack pathogens present in their parent have often been selected and named as cultivars. Thus, using morphological traits, it can be difficult to distinguish between many citrus cultivars. There are many examples of such cultivars, including scions such as many sweet orange cultivars, most grapefruit selections, and rootstocks such as many cultivars of trifoliate orange. Identification of these scion cultivars in nursery situations is particularly difficult because some cultivars are distinguishable only by fruit traits, and citrus trees usually do not bear fruit until 3–4 years after planting. It is also difficult to distinguish some rootstock cultivars after trees are planted in the field. This may become an issue if a grower suspects that a nursery sold trees on a rootstock other than that ordered. The ability to identify citrus cultivars using a small amount of leaf or other vegetative tissue would be helpful in protecting the rights of citrus breeders, growers and nurseries.

A variety of methods have been used for citrus cultivar identification (Roose 1988; Deng et al. 1995; Kijas et al. 1995; Luro et al. 1995). Isozyme analysis is useful for distinguishing cultivars that have been derived by sexual reproduction, but does not generally distinguish between cultivars that have differentiated by mutation (Roose 1988; Herrero et al. 1996). Restriction fragment

length polymorphisms (RFLPs) are highly polymorphic in citrus (Roose 1988). However, because a mutational event that results in the novel phenotypic characteristics of the derived cultivar may affect a very small proportion of the genome, perhaps only a single nucleotide in some cases, it is unlikely that such events can be detected efficiently by RFLP analysis using DNA probes randomly selected from the genome. Random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al. 1990) analysis is more efficient than RFLP analysis in cultivar identification (Santos et al. 1994; Rajapakse et al. 1995). In citrus, RAPD analysis has been used to identify chimeras (Sugawara and Oowada 1995) and lemon cultivars (Deng et al. 1995). However, because RAPD analysis does not target those rapidly evolving sequences that may be most likely to differ between mutationally derived cultivars, the detection of polymorphic RAPD markers may require PCR amplifications with many different primers. For example, Sugawara and Oowada (1995) found only three primers that were useful to identify two experimentally produced chimeras after they tested 124 primers. No differences were detected among six phenotypically diverse sweet orange cultivars after more than 100 decamer primers were tested (Xiao and Gmitter, personal communication). Mulcahy et al. (1993) showed that apple cultivars could be identified using RAPD markers but that the sports, which originated from one cultivar by mutation, were all identical to the ancestral cultivar.

Inter-simple sequence repeat (ISSR) amplification 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 anchored at the 3′ or 5′ end by 2–4 arbitrary, often degenerate, nucleotides. These primers target simple-sequence repeats (microsatellites) that are abundant throughout the eukaryotic genome (Tautz and Renz 1984; Kijas et al. 1995) and evolve rapidly (Levinson and Gutman 1987), but do not require prior knowledge of DNA sequence for primer design. 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 analysis (Wolff et al. 1995). ISSR analysis has been used to assess genetic diversity in dent corn and popcorn (Kantety et al. 1995) and in Douglas fir and sugi (Tsumura et al. 1996), as well as to identify cultivars of chrysanthemum (Wolff et al. 1995) and of oilseed rape (Charters et al. 1996).

In the present paper, we report the use of ISSR markers to fingerprint closely related citrus cultivars from six cultivar groups. Many of these cultivars belonging to the same cultivar group are difficult to distinguish with other molecular-marker techniques. Furthermore, we will present the results of RFLP analysis by using specific ISSR markers as probes to verify the genetic basis of ISSR markers.

Materials and methods

Plant materials

Leaves from 94 trees of 68 cultivars were sampled from the Citrus Clonal Protection Program collection located at the University of California, Lindcove Research and Extension Center, Exeter, California, and from the Citrus Variety Collection at the University of California, Riverside, California, USA. Additional samples of some cultivars were collected from commercial nurseries or obtained from Dr. W. S. Castle, Lake Alfred, Florida (Table 1). In this paper, we consider nucellar and old-line sources of the same cultivar as duplicate samples. After all of these samples were tested with all primers, a second DNA sample was extracted from the leaves collected from a different source tree in Riverside to test the repeatability and reliability of ISSR markers. Such samples included all sweet orange, except 'Tarocco' blood orange (Thermal source), grapefruit and trifoliate orange cultivars that had different fingerprint patterns in the previous experiment. Additionally, 15 progeny trees derived from a cross between 'Ruby' blood orange and 'Flying Dragon' trifoliate orange were sampled to test the inheritance of one ISSR marker unique to 'Ruby' blood orange.

DNA extraction and ISSR analysis

Total DNA extraction, ISSR-PCR amplification, electrophoresis, and silver staining were according to the protocols described previously (Fang et al. 1997).

RFLP analysis

Three ISSR markers, i.e., $(AG)_8YT$ 1320 bp, $(AG)_8YC$ 560 bp, and $(GA)_8YG$ 600 bp, that differentiated 'Moro' blood orange or 'Newhall' navel orange were excised from the lanes of 'Newhall' navel on polyacrylamide gels. A PCR amplification was performed with the original primer using the excised band as template DNA. The PCR products were separated on a polyacrylamide gel as above. The target band was excised again from the gel because a few products were observed in addition to the band of interest. By using this excised band as template, a PCR amplification was conducted again, and the PCR products were electrophoresised on a polyacrylamide gel. Only one intense band was observed on the gel, and this band was used as a probe for RFLP analysis. DNA from four sweet orange cultivars, i.e., 'Tarocco' blood (Thermal source), 'Lane late' navel, 'Newhall' navel and 'Moro' blood, were digested with 16 restriction endonucleases. The DNA digestion, electrophoresis, Southern transfer, probe labeling, hybridization and autoradiography were according to Jarrell et al. (1992).

Results and discussion

ISSR amplification

Forty six primers were initially tested using citrus DNA (Table 2). Fourteen primers amplified no products at all. Thirteen of them were based on $(AT)_n$, $(TA)_n$ or $(TC)_n$ repeats, each anchored by various nucleotides. Possibly, this indicates that the citrus genome lacks, or else has very few of, these three microsatellites, although Wang et al. (1994) reported that $(AT)_n$ was the most abundant microsatellite in plant nuclear genomes.

Table 1 Citrus cultivars evaluated with ISSR markers amplified using 22 primers

Cultivar	Tree location[a]
Valencia oranges (*Citrus sinensis* (L.) Osbeck)	
Campbell nuc.[b]	L
Campbell (old line)	L
Cutter nuc.	L
Frost nuc.	L
Olinda (old line)	L
Olinda nuc.	L
Olinda	R
Midknight	L
Delta	L
Rhode Red	R
Blood oranges (*C. sinensis*)	
Ruby	L
Ruby	R
Moro nuc.	L
Sanguinelli nuc.	L
Tarocco nuc.	L
Tarocco nuc.	R
Tarocco (Thermal source)[c]	R
Navel oranges (*C. sinensis*)	
Carter (old line)	L
Frost nuc.	L
Fisher (old line)	L
Rocky Hill (old line)	L
Cluster	L
Dream nuc.	L
Lane Late	L
Lane Late	R
Leng	L
Atwood (old line)	L
Parent Washington	L
Parent Washington	R
Gillette (old line)	L
Eddy (old line)	L
Newhall (old line)	L
Thomson (Zimmerman)	L
Thomson (Sheldon)	L
Fukumoto	L
Fukumoto	R
Rio Grande nuc.	L
Cara Cara pink	L
Dry navel	R
Corrigated Thomson	R
Seedy Washington	R
Grapefruits (*C. paradisi* Macf.)	
Redblush	L
Redblush	R
Marsh Reed	L
Star Ruby	L
Star Ruby	R
Rio Red	L
Rio Red	R
Henderson Ruby	L
Ray Ruby	L
Flame	L
Lemons (*C. limon* (L.) Burm. f.)	
Frost Eureka nuc.	L
Variegated pink flesh Eureka	L
Frost Lisbon nuc.	L
Monroe Lisbon (old line)	L
Limoneira 8A Lisbon	L
Limoneira 8A Lisbon	R
Seedless Lisbon	L
Mandarins (*C. reticulata* Blanco)	
Minneola (old line)	L
Minneola nuc.	L
Minneola	L
Ellendale	L
Clementine Algerian	L
Clementine Monreal	L
Murcott	L
W. Murcott	L
Koster	R
Frost Owari satsuma	L
Nepolitana satsuma	L
Dobashi Beni satsuma	L
Okitsu Wase satsuma	L
Kawano Wase satsuma	R
Citranges (*C. sinensis* × *Poncirus trifoliata* (L.) Raf.)	
Troyer	N1
Troyer	L
Troyer	N2
Troyer	R
C-32	R
C-32	L
C-35	L
C-35	R
Carrizo	N2
Carrizo	N1
Kuharske Carrizo (seedling)[c]	Florida
Carrizo (seedling)	Florida
Carrizo	N3
Carrizo	L
Carrizo	R
Benton	L
Trifoliate oranges (*P. trifoliata*)	
Rubidoux	L
Rich 16–6	L
Rich 16–6	R
Pomeroy	R
Flying Dragon	L
Flying Dragon	R

[a] L = Lindcove, R = Riverside, N1 = commercial nursery #1, etc.
[b] nuc. = nucellar
[c] Considered a separate cultivar in this study

Alternatively, lack of amplification products may be due to the self-complementary nature of $(AT)_n$ or $(TA)_n$ primers. $(TCC)_5RG$ produced no amplification products either, although the other three primers with $(TCC)_5$ generated excellent results. Ten primers produced smears or fuzzy patterns that could not be scored. We tried adjusting annealing temperatures and changing amplification cycles, but no significant improvements were made for these ten primers. We believe that the poor results obtained were due either to characteristics of the primers or to the relative abundance of priming sites in the citrus genome. The other 22 primers involving seven core repeats gave clear, species-specific fingerprint patterns with all samples (Figs. 1, 2). The amplified fragment sizes ranged from

Table 2 ISSR primers screened with citrus DNA

Primers[a]	Results[b]	Primers	Results	Primers	Results
$HVH(TG)_7T$	45	$(AC)_8YA$	55	$(TG)_8RT$	Smeared or fuzzy
$VHVG(TG)_7$	53	$(AC)_8YG$	51	$(TG)_8RC$	Smeared or fuzzy
$BDB(TCC)_5$	28	$(GT)_8YC$	54	$(TG)_8RA$	Smeared or fuzzy
$HVH(TCC)_5$	46	$(GT)_8YG$	60	$(TG)_8RY$	Smeared or fuzzy
$DBDA(CA)_7$	45	$VBV(AT)_7$	No product	$HBH(AG)_7$	Smeared or fuzzy
$HVH(CA)_7T$	66	$(AT)_8YA$	No product	$BHB(GA)_7$	Smeared or fuzzy
$BDB(CA)_7C$	45	$(AT)_8YC$	No product	$(CA)_8RT$	Smeared or fuzzy
$DBD(AC)_7$	32	$(AT)_8YG$	No product	$(CA)_8RC$	Smeared or fuzzy
$(TCC)_5RY$	42	$(TA)_8RT$	No product	$(GT)_8YT$	Smeared or fuzzy
$(CA)_8RG$	58	$(TA)_8RC$	No product	$(GT)_8YA$	Smeared or fuzzy
$(CA)_8RY$	54	$(TA)_8RG$	No product		
$(GA)_8YT$	60	$BVB(TA)_8$	No product		
$(GA)_8YC$	97	$(TC)_8RA$	No product		
$(GA)_8YG$	65	$(TC)_8RT$	No product		
$(AG)_8YT$	97	$(TC)_8RG$	No product		
$(AG)_8YC$	73	$(TC)_8BC$	No product		
$(AG)_8YG$	63	$HVH(TC)_7$	No product		
$(AC)_8YT$	40	$(TCC)_5RG$	No product		

[a] R = purine, Y = pyrimidine, B = non-A, D = non-C, H = non-G, V = non-T
[b] Number of fragments in a navel orange cultivar

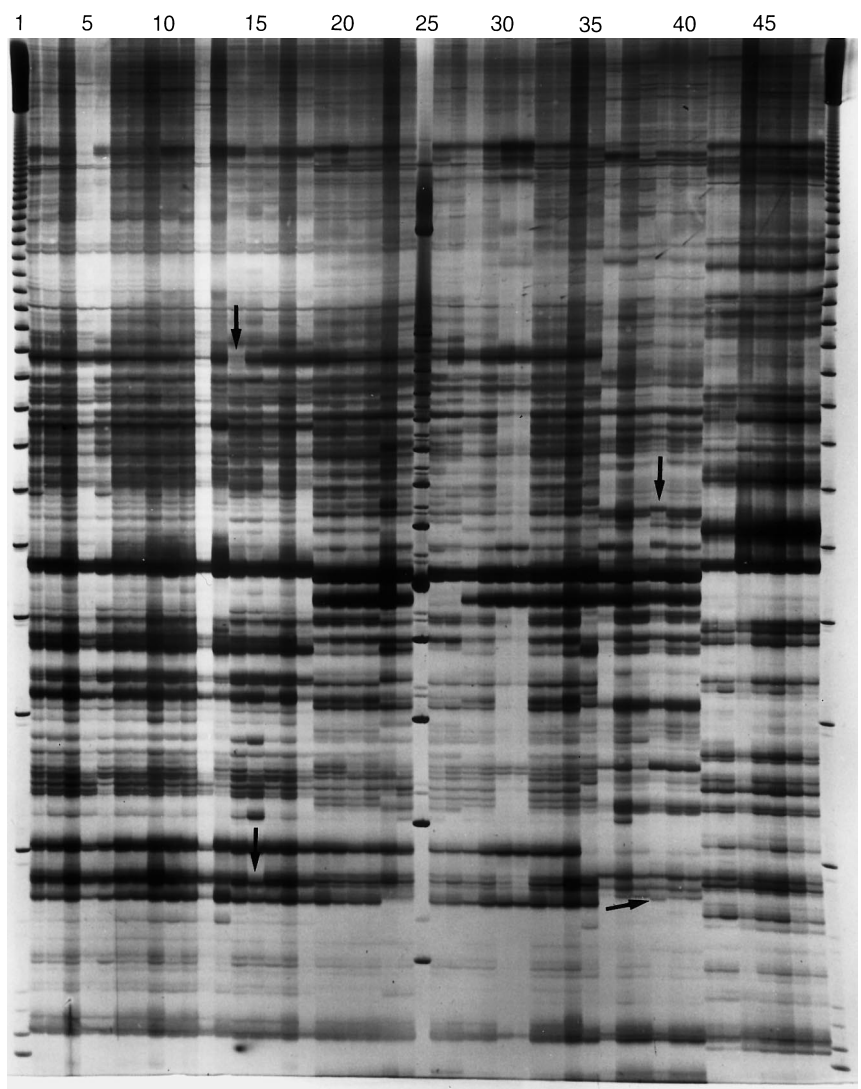

Fig. 1 ISSR profiles amplified from DNA of citrus cultivars using primer $(AG)_8YT$. *Lanes 1 and 49:* 123-bp ladder; *lane 25*: 100-bp ladder (the smallest band is 300 bp); *lanes 2–11*: valencia oranges; *lanes 12–18*: blood oranges; *lanes 19–24 and 26–35*: citranges; *lanes 36–41*: trifoliate oranges; *lanes 42–48*: lemons. The order of lanes within each cultivar group is the same as that in Table 1. *Arrows* indicate differences between cultivars mentioned in the text

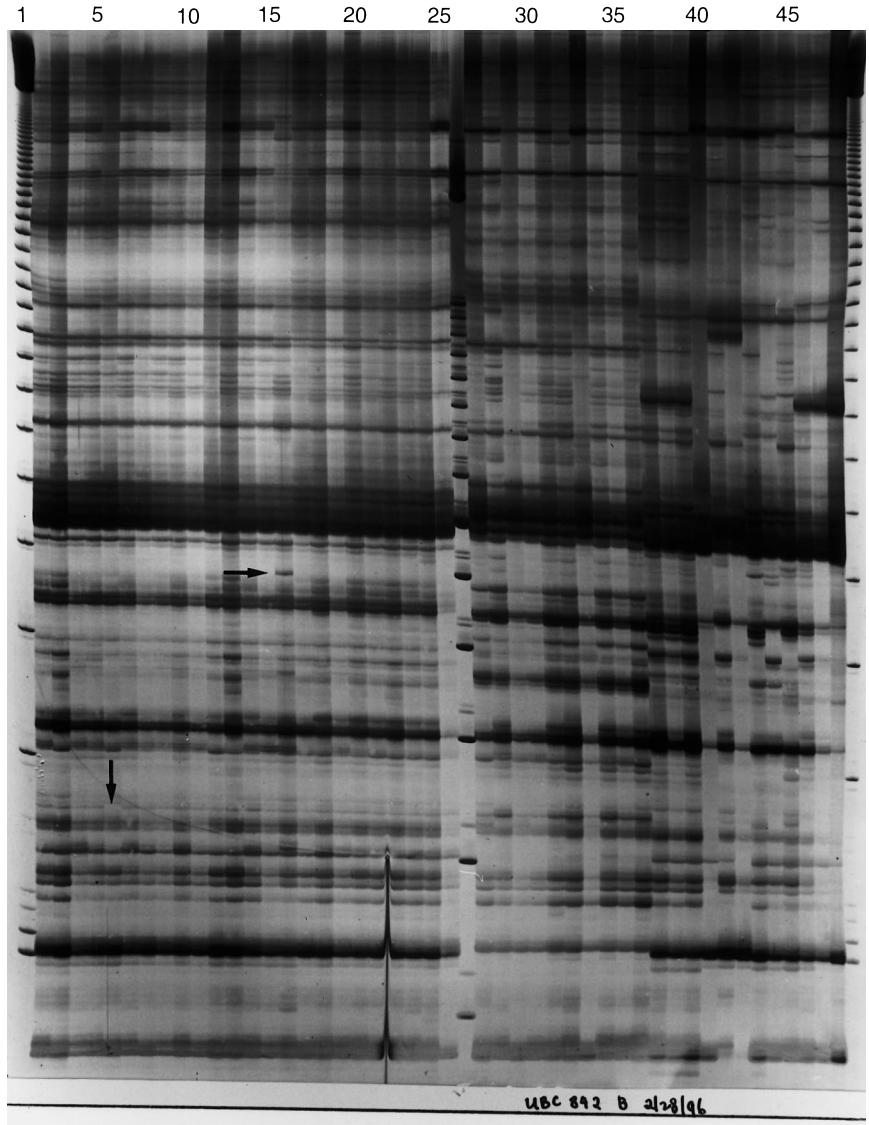

Fig. 2 ISSR profiles amplified from DNA of citrus cultivars using primer $(GA)_8YG$. *lanes 1 and 49*: 123-bp ladder; *lane 26*: 100-bp ladder (the smallest band is 200 bp); *lanes 2–25*: navel oranges; *lanes 27–36*: grapefruits; *lanes 37–48*: mandarins. The order of lanes within each cultivar group is the same as that in Table 1. *Arrows* indicate differences between cultivars mentioned in the text

80 to 3500 bp with the scoreable region being from 100 to 2500 bp. Fingerprint patterns consisted of 28–97 fragments with an average of 56 fragments per primer in navel orange and a similar number in most other cultivars. Though we did not do an extensive comparison between 3′- and 5′-anchored primers using the same core repeat, our results indicate that 5′-anchored primers generally have more specificity and thus generate fewer but larger fragments than 3′-anchored ones. The mean fragments per primer in navel orange were 45 and 62 for 5′ and 3′-anchored primers, respectively. These results may be useful for selecting ISSR primers for other species.

We systematically studied many factors both of PCR amplification and of staining to achieve clear fingerprint patterns. High-quality DNA template was essential to obtain a large number of well-resolved fragments. RNA in DNA template preparations interfered with the PCR reaction and reduced the number of fragments that could be scored. DNA suitable for RFLP analysis is suitable for ISSR-PCR amplification. Inclusion of formamide in the PCR reaction was essential for repeatable amplification, and reduced background and smearing on gels. Formamide may influence primer-template annealing and melting temperatures (Tsumura et al. 1996). If formamide was excluded from the reaction mixture, no or fewer fragments were detected. However, formamide concentrations of 3% or above inhibited amplification completely; 2% formamide in the reaction mixture generally gave good results.

Variation between species

Each of the 22 primers tested discriminated among all five species. Each species had at least three unique fragments with each primer (Figs. 1, 2). Citranges are

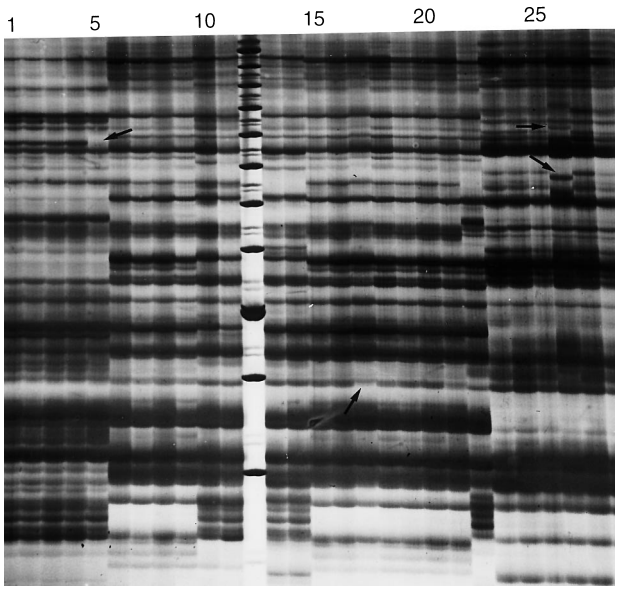

Fig. 3 ISSR profiles amplified from DNA of citrus cultivars using primer BDB(CA)$_7$C showing markers unique to 'Tarocco' blood orange (Thermal source) (*lane 5*), to 'Kuhaske Carrizo' citrange (*lane 17*), and to 'Pomeroy' trifoliate orange (*lane 26*). *Lane 1*: 'Moro' blood orange; *lane 2*: 'Sanguinelli' blood orange; *lanes 3–4*: two samples of 'Tarocco' blood orange; *lanes 6–11 and 13–22*: citranges; *lane 12*: 100-bp ladder (the smallest band is 500 bp); *lanes 23–28*: trifoliate oranges. The order of lanes within the citrange and trifoliate orange groups is the same as that in Table 1. *Arrows* indicate differences mentioned in the text

hybrids between sweet orange and trifoliate orange, and their fingerprint patterns include fragments also present in sweet orange or trifoliate orange (Figs. 1, 3).

Identification of closely related cultivars within a species

Sweet oranges

Sweet orange is the most important citrus species in the world in terms of acreage and production. Furthermore, nearly all commercially important sweet orange cultivars originated through mutations which alter horticultural characters, mostly fruit traits (Hodgson 1967). Consequently, many sweet orange cultivars can be distinguished only by fruit traits. To assess the power of ISSR markers to differentiate among sweet orange cultivars, we analyzed 41 samples of 33 cultivars that belong to three groups, i.e., Valencia, blood and navel, based on fruit traits. All of these cultivars had almost the same ISSR fingerprints, and isozyme and RFLP profiles as well (Roose 1988). This further supports the view that a majority of sweet orange cultivars derived from a single ancestor by mutation. However, 14 cultivars differed from the basic fingerprint consisting of about 1230 fragments by 1–4 ISSR markers (Table 3).

Among the seven Valencia orange cultivars, only 'Midknight' differed from the others by loss of a 590-bp fragment amplified by primer HVH(CA)$_7$T (Fig. 4 A).

Among the blood oranges, four of the five cultivars tested had unique fingerprints for 1–3 fragments that distinguished them from all other sweet orange cultivars. Only 'Tarocco' blood orange from both Riverside and Lindcove could not be distinguished from the basic sweet orange fingerprint pattern. Primers DBDA(CA)$_7$ and BDB(CA)$_7$C revealed differences (Fig. 3) between a 'Tarocco' blood orange (Thermal source) and other cultivars including 'Tarocco' blood orange from both Lindcove and Riverside. With both

Table 3 ISSR markers that differentiated sweet orange cultivars

Cultivar	Primer: fragment size[a]
Midknight valencia	HVH(CA)$_7$T: − 590
Ruby blood	(TCC)$_5$RY: + 550, (GA)$_8$YT: − 390, (GA)$_8$YG: −1150
Moro blood	(AG)$_8$YT: − 1320, (GA)$_8$YG: + 650
Sanguinelli blood	(AG)$_8$YT: − 350
Tarocco blood (Thermal source)	DBDA(CA)$_7$: −1080, BDB(CA)$_7$C: −1080, HVH(CA)$_7$T: −470
Fisher navel	(GA)$_8$YC: + 980
Cluster navel	(GA)$_8$YG: −340, (GA)$_8$YC: + 410
Dream navel	(GA)$_8$YC: + 1380
Parent Washington navel (Riverside)	(AG)$_8$YC: + 480, (GA)$_8$YC: + 430
Gillette navel	(GA)$_8$YC: + 530
Newhall navel	(AG)$_8$YC: + 500, + 560, -570, (GA)$_8$YG: + 600
Thomson (Sheldon) navel	VHVG(TG)$_7$: + 650
Fukumoto navel	(GA)$_8$YC: + 440, + 450, + 410
Corrigated Thomson navel	(GA)$_8$YC: + 400, + 1180

[a] − 590 indicates absence of a 590-bp fragment common to all other sweet orange cultivars, and + 590 indicates a 590-bp fragment unique to this cultivar

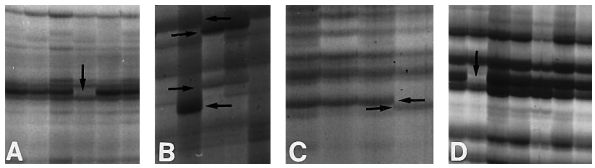

Fig. 4A–D ISSR profiles amplified from DNA of citrus cultivars using different primers. **A** Primer HVH(CA)$_7$T. *lane 1*: 'Cutter' nucellar valencia orange; *lane 2*: 'Frost' nucellar valencia orange; *lane 3*: Olinda valencia orange; *lane 4*: 'Midknight' valencia orange; *Lane 5*; 'Delta' valencia orange; *Lane 6*: 'Rhode Red' valencia orange. *Arrow* indicates the absence of 590-bp fragment in 'Midknight' valencia orange. **B** Primer (AG)$_8$YC. *Lanes 1–2*: Parent 'Washington' navel orange from Lindcove and Riverside, respectively; *lanes 3–4*: 'Newhall' navel orange from Lindcove and Riverside, respectively; *Lane 5*: 'Thomson (Zimmerman)' navel orange. *Arrows* indicate four polymorphic fragments, i.e. 480, 500, 560 and 570 bp. **C** Primer (AG)$_8$YT. *Lane 1*: 'Rio Red' grapefruit from Lindcove; *lane 2*: 'Rio Red' grapefruit from Riverside; *lane 3*: 'Rio Red' grapefruit from a tree in a commercial grove in Riverside; *lane 4*: 'Star Ruby' grapefruit. *Arrows* indicate two polymorphic fragments, i.e. 850 and 860 bp. **D** Primer (GA)$_8$YG. *Lanes 1–7*: seven lemon cultivars. The lane order is same as that in Table 1. *Arrow* indicates the absence of 520-bp fragment in 'Variegated pink flesh' Eureka lemon

primers, 'Tarocco' blood orange (Thermal source) lacked a 1080-bp fragment which was shared by all other sweet orange cultivars. This result implies that these two primers revealed the same mutation event. Moreover, HVH(CA)$_7$T highlighted another difference, i.e., absence of a 470-bp fragment in 'Tarocco' blood orange (Thermal source). 'Tarocco' blood orange (Thermal source) is less thorny, and has deeper red flesh color than the 'Tarocco' blood orange trees from both Riverside and Lindcove. We have shown that this character divergence is accompanied by molecular divergence.

Among the 21 navel orange cultivars, nine had unique fingerprint patterns. A 410-bp fragment amplified by primer (GA)$_8$YC was observed only in 'Cluster' navel and in 'Fukumoto' navel. 'Newhall' navel had three unique fragments but lacked one fragment which was shared by all other sweet orange cultivars. The Parent 'Washington' navel from Riverside had two unique fragments not amplified from the Parent 'Washington' navel sampled from Lindcove (Fig. 4 B). This is the only case in which replicate samples of the same cultivar from different locations had different ISSR fingerprint patterns. This result suggests that mutation occurred in at least one of them although horticultural traits are not known to differ between them. Parent 'Washington' navel orange exhibits a remarkable degree of somatic instability. Since 1874, when three Parent 'Washington' navel orange trees were planted in Riverside, California, many mutations have been described, and nearly all of the navel orange cultivars in the USA are known or believed to have originated from two of these Parent 'Washington' navel orange trees (one tree died shortly after it was planted), either as bud or limb sports mutations or as nucellar seedlings (Hodgson 1967).

Grapefruits

The origin of true grapefruit cultivars is well-documented and it is clear that all were derived from the same ancestral tree by mutation (Gmitter 1995). Among the seven cultivars studied in this experiment, only 'Rio Red' was differentiated from the others. In the amplification products of primer (AG)$_8$YT, 'Rio Red' had a unique 860-bp fragment and lacked a 850-bp fragment that was present in all other grapefruit cultivars (Fig. 4 C).

Lemons

Lemon cultivars are evidently more divergent from one another than sweet orange or grapefruit cultivars because a much higher proportion of lemon cultivars had unique fragments. Possibly, lemon cultivars have a polyphyletic origin. A total of 12 polymorphic fragments generated by seven primers were detected among seven samples of six lemon cultivars (Table 4, Fig. 4 D). All of the cultivars could be distinguished from one another except that 'Monroe' Lisbon was identical to 'Limoneira 8A' Lisbon. 'Frost' Lisbon was the most distinct, having two unique fragments and lacking five common ones. The two samples of 'Limoneira 8A' Lisbon were identical.

Mandarins

Mandarin cultivars showed much more diversity than those within the other species (Fig. 2). However, the five mutationally derived satsuma cultivars were identical to one another, as were the two 'Clementine' cultivars. 'Koster' originated from 'Ellendale' via mutation. They had the same fingerprints with most of the primers; however, HVH(TG)$_7$T, (AG)$_8$YC and (GT)$_8$YA revealed differences between 'Koster' and 'Ellendale'. The three sources of 'Minneola' could not be distinguished from each other except that 'Minneola' nucellar had a unique 1000-bp fragment in HVH(TCC)$_5$-generated fingerprints. Although their names imply a close relationship, 'W. Murcott' and 'Murcott' mandarins are only distantly related. Any one of the 22 primers tested can discriminate between them.

Trifoliate oranges

Among the trifoliate orange cultivars, ISSR markers suggest that 'Pomeroy' is quite divergent from

Table 4 ISSR markers that differentiated lemon cultivars

Cultivar	$(CA)_8RG$		$(TCC)_5RY$			VHVG$(TG)_7$	HVH$(CA)_7$T	$(AG)_8$YT	$(AG)_8$YC			$(GA)_8$YG
	1000 bp	1020	880 bp	890	900	900 bp	500 bp	450 bp	630 bp	1500	1520	520 bp
Frost Eureka	+	+	−	−	+	+	+	+	−	−	+	+
Variegated pink flesh Eureka	+	+	−	+	+	−	+	+	−	+	+	−
Frost Lisbon	+	−	−	+	−	+	−	−	+	−	−	+
Monroe Lisbon	+	+	+	−	−	+	+	+	−	−	+	+
Limoneira 8A Lisbon	+	+	+	−	−	+	+	+	−	−	+	+
Seedless Lisbon	+	+	−	−	+	+	+	+	−	−	+	+

'Rubidoux', 'Rich 16-6', and 'Flying Dragon' (Figs. 1, 3). Ten of twenty two primers produced different fingerprint patterns for 'Pomeroy', with each primer generating at least one unique fragment. No marker was found to distinguish among the other three cultivars. 'Pomeroy' is in the large-flowered group, while the other three cultivars are in the small-flowered group. A more comprehensive study on fingerprinting trifoliate orange accessions was conducted by us in a separate experiment (Fang et al. 1997).

Citranges

Among the citranges, each cultivar had a distinct pattern with each primer, except that 'Troyer' was indistinguishable from 'Carrizo'. Difficulty in distinguishing 'Troyer' from 'Carrizo' citranges is not surprising since these two cultivars originated from a single hybrid seedling within this century (Savage and Gardner 1965). These cultivars had identical fingerprint patterns, further supporting their derivation from the same hybrid seedling. No differences were detected among four sources of 'Troyer' and six sources of 'Carrizo'. 'Kuharske Carrizo' from Florida differed from the standard 'Carrizo' accessions with primers BDB$(CA)_7$C (Fig. 3) and $(GA)_8$YG. The distinct pattern of 'Kuharske Carrizo' is interesting because this accession has been identified as having better citrus nematode resistance than other sources of 'Carrizo' (Kaplan 1992). The material we tested originated from a seedling source, but the very high rate of nucellar embryony in 'Carrizo' citrange, and the fact that only two differences were detected out of about 1230 ISSR fragments, make it very unlikely that the seedling was of zygotic origin.

Repeatability of ISSR markers

Reliability and repeatability are essential for a technique to be used for cultivar identification. In the current study, we thoroughly tested the repeatability of ISSR markers by using DNA samples of the same cultivar in different locations, or DNA extracted from different leaves of the same tree, or by performing separate PCR runs. All of the differences described above were re-tested with DNA extracted from different trees of the same cultivar and all fingerprint patterns were confirmed. No new differences were detected in the second experiment. Except for the Parent 'Washington' navel orange samples from Riverside and Lindcove, replicate samples had identical fingerprints with all primers for the other 17 cultivars sampled from more than one location. All of these indicate that ISSR markers are highly reproducible. The primers used could amplify DNA from virtually any eucaryotic organism (Zietkiewicz et al. 1994), so contamination of

leaf samples with insects or perhaps fungi could potentially cause differences between samples. In citrus, careful washing of leaf samples appears adequate to minimize this problem as shown by confirmation of all of the differences reported here using DNA extracted from separate leaf samples collected from a different source tree. Over 99% of the scored fragments can be repeated across DNA samples of the same cultivar and across separate PCR runs. Only very faint fragments were not reproducible and such fragments were not scored or else were eliminated from the data set in this study.

Verification of the genetic basis of ISSR markers

In order to verify the genetic basis for ISSR markers, we used three ISSR markers, i.e., $(AG)_8YT$ 1320 bp, $(AG)_8YC$ 560 bp and $(GA)_8YG$ 600 bp, that differentiated 'Moro' blood or 'Newhall' navel orange as probes to hybridize with the Southern blots of DNA from four sweet orange cultivars digested with 16 restriction endonucleases. Three enzymes, i.e., *Bfa*I, *Dra*I and *Xha*I, produced polymorphisms between four cultivars when marker $(AG)_8YT$ 1320 bp was employed as a probe. When marker $(AG)_8YC$ 560 bp was used as a probe, six enzymes, i.e., *Bfa*I, *Dra*I, *Hae*III, *Hin*dIII, *Mbo*I and *Msp*I, revealed polymorphisms between four cultivars (Fig. 5). *Bfa*I, *Bsto*I, *Dra*I, *Eco*RI, *Mbo*I, *Msp*I, *Pal*I, and *Rsa*I, produced RFLPs when marker $(GA)_8YG$ 600 bp was used as a probe. Overall, each of the ISSR-derived probes uniquely distinguished each of the four cultivars when DNA was digested with one or more restriction endonucleases. Although we only used four sweet orange cultivars in our RFLP analysis, our results clearly showed that ISSR-derived probes revealed many polymorphic RFLP markers for the identification of mutationally derived citrus cultivars.

The $(TCC)_5RY$-generated marker [$(TCC)_5RY$ 550 bp] that was unique to 'Ruby' blood orange was also present in 'C-35' citrange, a hybrid of 'Ruby' blood orange and trifoliate orange. This observation suggested that this unique marker is inherited. We further tested the inheritance of this marker among 15 progeny trees derived from a cross between 'Ruby' blood orange and 'Flying Dragon' trifoliate orange. This marker was present in six progeny trees and absent in the other nine trees, suggesting that it is inherited in a Mendelian fashion. Tsumura et al. (1996) reported that ISSR markers are generally inherited as dominant markers in Douglas fir and sugi. In citrus, ISSR markers are also usually inherited as dominant markers (data not shown).

We have not explored the molecular basis of variation in ISSR markers. Some differences involve gain of a marker and some involve loss. Variation could be due to mutations in length of the simple-sequence repeat, to mutations that alter anchored nucleotides, or to insertion or deletion mutations in the sequence between the primer sites. Although much remains unknown about ISSR markers, they appear to offer a very powerful and efficient system for distinguishing among citrus cultivars, particularly for those that have diverged by mutation.

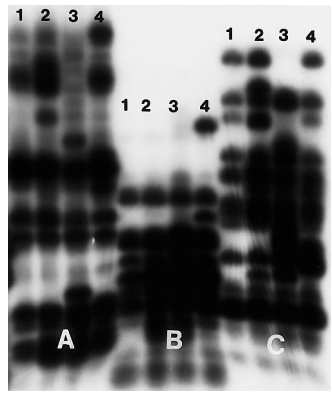

Fig. 5 Southern-blot analysis of sweet orange DNA using excised and reamplified ISSR marker $(AG)_8YC$ 560 bp as probe. *1*: 'Tarocco' blood orange (Thermal source); *2*: 'Lane late' navel orange; *3*: 'Newhall' navel orange; *4*: 'Moro' blood orange. *A*: *Hae*III; *B*: *Mbo*I; *C*: *Bfa*I.

In conclusion, ISSR markers offer great potential for differentiating closely related citrus cultivars. We have distinguished more sweet orange cultivars with ISSR markers than with any other molecular-marker system. To the best of our knowledge, ISSR is the first marker system that differentiates certain grapefruit cultivars. More polymorphic markers were identified between sweet orange cultivars when ISSR markers were used as probes in RFLP analysis. It may be possible to differentiate most mutationally derived citrus cultivars by studying more ISSR primers and by using ISSR markers as probes in RFLP analysis. It will be interesting to investigate the molecular basis of differences between two cultivars by cloning and sequencing the regions that differ between them. Our recent studies on the inheritance of ISSR markers show that such markers have great potential uses in citrus genomic mapping.

Acknowledgements We thank the California Citrus Nursery Advisory Board for support.

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