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Identification of Zygotic and Nucellar Individuals Produced from Several Citrus Crosses Using SSRs Markers

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Abstract

Turkey is an important citrus-producing country. However, new cultivars are needed to sustain citrus production and ensure its competitiveness against other crops. There are currently several citrus breeding programs that aim to help overcome the lack of local commercial varieties and to contribute to Turkey's competitive capacity in the citrus market. In this study, we report the utilization of molecular markers in one such breeding program. Simple sequence repeat (SSR) markers were employed to eliminate nucellar individuals from a hybrid population produced by crossing. The crosses included 'Fremont' and 'Robinson' mandarins as the female parents and 'Midknight Valencia', 'Rhode Red Valencia', and 'Valencia Late' oranges and 'Rio Red' grapefruit cultivars as the male parents. Seedlings with the same banding patterns as the female parent were identified as nucellar seedlings by 11 SSR primers. Primers AG14 and TAA03 were found to be more effective at identifying zygotic individuals than other primers. 'Fremont' and 'Robinson' mandarins produced 36.91% and 31.09% of nucellar seedlings, respectively. As a pollen parent, 'Rio Red' grapefruit had a higher ratio of zygotic seedlings compared to 'Midknight Valencia', and can be recommended in breeding programs. Comparative analysis of different citrus fruits in the breeding programs allowed us to design an efficient hybridization scheme for this study.

Key words: breeding, pollen parent, nucellar seedling, molecular marker

Introduction

Citrus, a major fruit crop, is grown throughout the tropical and subtropical regions of the world. Most of the citrus cultivars have resulted from natural hybridization and mutation (Hodgson, 1967). Most citrus breeding programs have relied mainly on classical breeding practices, which are based on making controlled crossings and selecting superior individuals. One of the major problems of the classical breeding programs when using these cultivars as a seed parent is polyembryony, when one seed may contain two or more embryos of zygotic or nucellar origin (Soost and Roose, 1996). Nucellar embryony, which occurs commonly in citrus species, creates a serious problem for cross breeding studies as it produces a large number of asexual embryos, greatly limiting the genetic variability obtained by controlled pollination. In a seed, adventive (extra) embryos that develop almost entirely from nucellus cells contain the same genetic material as the mother plant and can create a number of problems. Firstly, most polyembryonic cultivars produce only a small number of crosses. Secondly, it is often difficult to distinguish nucellar and zygotic seedlings at an early stage (De Lange and Vincent, 1977). However, discrimination of nucellar seedlings at an early stage is essential to avoid the 5-10 years of expense that accompany the unwarranted growth and maintenance of nucellar seedlings that are genetically identical to seed parents .

Polyembryony is caused by a number of factors including the type of pollinator, the amount and viability of available pollen, plant nutrition, air temperature, environmental and soil humidity, and wind speed (Spiegel-Roy *et al*., 1977; Nishiura and Iwasaki, 1980; Soost *et al*., 1980; Nakatani *et al*., 1984; Khan and Roose, 1988; Moore and Castle, 1988; Roose and Traugh, 1988; Soares-Filho *et al*., 1995). Dhillon *et al*. (1993) reported that adventitious embryos developed into globular or early cotyledon stages in the absence of pollination, but that the embryos required the endosperm in order to grow, making pollination and fertilization necessary for the development of the polyembryonic seeds. Consequently, some factors that influence pollination, fertilization or seed development will also affect the percentage of polyembryony and embryo number per seed. Garcia *et al*. (1999) suggested that when the zygotic embryo is a hybrid, it may be more vigorous and compete better with nucellar embryos, whereas zygot-

ic embryos produced by self-pollination are less vigorous and may not be competitive with nucellar embryos.

Many studies aimed at separating the different types and varieties of citrus, as well as those aimed at separating nucellar and zygotic plants, started by utilizing morphological characterization (Furr and Reece, 1946; Cameron, 1979), spectrophotometer (Pieringer and Edwards, 1967) and chromatography techniques (Albach and Redman, 1969; Stanley and Jurd, 1971; Tatum *et al*., 1974; Weinbaum *et al*., 1982). Several biochemical methods, including enzymatic darkening due to polyphenols (Esen and Soost, 1974), have also been used. None of these methods efficiently confirmed the identity of true nucellar seedlings (Ruiz *et al*., 2000; Tusa *et al*., 2002). Later, isozymes were employed in plant breeding (Iglesias *et al*., 1974; Moore and Castle, 1988; Ashari *et al*., 1988; Anderson *et al*., 1991). Nevertheless, because products of gene expression were used in those cases, results may be influenced by the environment or by the developmental stage of the plant and its organs, thus making this method unreliable for zygotic seedling identification. Recently, molecular marker techniques have been improved, enabling more specific and precise studies to be conducted. These new techniques are particularly advantageous for woody plants as they allow selection in much shorter time periods than previously possible.

The use of DNA polymorphisms for the identification of hybrid seedlings is important in citrus breeding programs as it accelerates the process of progeny screening. Among DNA-based methods, random amplified polymorphic DNA (RAPD) analysis is one of the most widely used for differentiating hybrids in citrus breeding programs (Bastianel *et al*., 1998; Vilarinhos *et al*., 2000). A number of recent studies have also described the use of SSR markers as an alternate method to distinguish sexual from nucellar citrus seedlings (Mullis *et al*., 1986; Ruiz *et al*., 2000; Oliveira *et al*., 2002; Rao *et al*., 2008; Shareefa *et al*., 2009).

In this report, we describe the identification of nucellar individuals within a population composed of F_1 populations of different citrus species and cultivars based on the analysis using SSR markers.

Materials and methods

Plant material

Controlled pollinations with pollens into flowers of trees growing in the Mustafa Kemal University experimental farm in Dörtyol, Hatay, Turkey (36° 09' E, 36° 51' N; altitude of 9 m) were conducted at full bloom. For crossing, the flowers of the balloon stage in the clusters of the mother parents were selected, and the others remained properly to avoid pollen contamination. Emasculation of male parts of the flowers was done with forceps carefully to avoid any injury of the stigma, and the all previously opened flowers and small, immature buds were removed manually. Stigmas were hand-pollinated with anthers from freshly opened flowers by a drawing brush. Immediately after pollination, the pollinated flowers were covered with cotton bags. Each pollination treatment was applied to flowers on three trees (about 100 flowers per treatment). The crossed flowers were marked with tag and tagging was continued up to harvesting of fruit, developed from crossing. Bags were removed 2 weeks after pollination. Fruits were harvested at maturity in the first week of December for Robinson cultivar, in the mid-December for Fremont cultivar and all fruits were individually weighed and seeds extracted. The mandarin cultivars 'Fremont' and 'Robinson' were used as female parents, and the orange cultivars 'Valencia Late', 'Midknight Valencia' and 'Rhode Red Valencia' with 'Rio Red' grapefruit were used as male parents.

A total of 500 seedlings were produced as a result of these crosses. For DNA extracting, the leaves were collected from seedlings with 9 months old.

DNA extraction and SSR analysis

Genomic DNA was extracted from young leaves using the CTAB method modified by Doyle and Doyle (1990). A total of 11 SSR primers (Tab. 1), as previously described by Kijas *et al*. (1997), were used to amplify the DNA. Polymerase chain reaction (PCR) amplifications were performed as described by Gulsen *et al*. (2005) with minor modifications. Each 15 µL amplification reaction consisted of 0.2 µg/µL BSA (bovine serum albumin), 200 µM total dNTP, 2.5 mM MgCl2, 1.33 mM each primer, 1 U of Platinum Taq polymerase (Invitrogen), 4.3 µL ddH2O and 20 ng DNA template. Amplification was performed in a DNA thermal cycler (Sensoquest Progen Scientific Ltd. Mexborough, South Yorkshire, UK) under the following conditions: 2 min at 94°C, followed by 5 cycles at 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, followed by 35 cycles for 1 min at 94°C, 1 min at 50°C and a final extension at 72°C for 1 min.

PCR products were mixed with 3 µL 5 x loading buffer (20 mL (40%) glycerol, 0.05 g Bromophenol Blue, 30 mL ddH2O) and analyzed by electrophoresis in 2% agarose gels under nondenaturing conditions in 1x TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, pH 8.3 and 25 µL (10 mg/mL) ethidium bromide). Five µl of 100 bp DNA ladder was used as size marker (Biorun, Nantes, France). Electrophoresis was also carried out on 10% PAGE (20 x 20 cm), at 115 V for 3 h. To identify hybrid and nucellar seedlings, DNA polymorphisms were analyzed by comparing the SSR markers produced by these seedlings with those generated by the parents.

Data analysis

Each band was scored as present (1) or absent (0), and data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package (Rohlf, 1993). A similarity matrix was constructed based

Tab. 1. SSR primer sequences used to identify zygotic Citrus progeny

No	Primer	Sequence	
1	TAA1	5'-GACAACATCAACAACAGCAAGAGC	3'-AAGAAGAAGAGCCCCCATTAGC
2	TAA3	5'-AGAGAAGAAACATTTGCGGAGC	3'-GAGATGGGACTTGGTTCATCACG
3	TAA41	5'-AGGTCTACATTGGCATTGTC	3'-ACATGCAGTGCTATAATGAATG
4	TAA45	5'-GCACCTTTTATACCTGACTCGG	3'-TTCAGCATTTGAGTTGGTTACG
5	TAA52	5'-GATCTTGACTGAACTTAAAG	3'-ATGTATTGTGTTGATAACG
6	AG14	5'-AAAGGGAAAGCCCTAATCTCA	3'-CTTCCTCTTGCGGAGTGTTC
7	ATC09	5'-TTCCTTATGTAATTGCTCTTTG	3'-TGTGAGTGTTTGTGCGTGTG
8	CAC33	5'-GGTGATGCTGCTACTGATGC	3'-CAATTGTGAATTTGTGATTCCG
9	CAGG9	5'-AATGCTGAAGATAATCCGCG	3'-TGCCTTGCTCTCCACTCC
10	CAT01	5'-GCTTTCGATCCCTCCACATA	3'-GATCCCTACAATCCTTGGTCC
11	CT02	5'-ACGGTGCGTTTTGAGGTAAG	3'-TGACTGTTGGATTTGGGATG

on Dice's coefficient (Dice, 1945), which considers only one-to-one matches between two taxa for similarity.

Results and discussion

Zygotic seedlings were determined by the presence of bands amplified specifically in the pollen parent from PCR using the 11 primers. Among the 233 F_1 plants obtained from the crosses with 'Fremont' mandarin as the female parent, 86 seedlings (36.91%) were identified as nucellar seedlings (Tab. 2). For 'Robinson' mandarin as the female parent, this ratio was 31.09% (83 nucellar seedlings out of 267 F_1 plants). Various levels of zygotic seedlings have also been observed in other citrus species and crosses. Frost and Soost (1967) reported zygotic frequencies of 78.7% and 14.0% for 'King' and 'Willowleaf' tangerines, respectively, using pollen from Poncirus trifoliata. Hearn (1973) reported that the selection of 'Mediterranean Sweet' orange produced 32% monoembryonic seeds and 62% zygotic seedlings when P. trifoliata pollen was used. Soost *et al*. (1980) found a zygote frequency of approximately 85% using 'King' as the female parent and pollen from 'Parson Special' tangerine. Several studies have shown that zygote frequency does not exceed 15%, depending on the species, and in some cases, only nucellar individuals are obtained (Cameron and Soost, 1980; Hirai *et al*., 1986; Ashari *et al*., 1988; Roose and Traugh, 1988). These results were similar to those reported by Moore and Castle (1988), who identified 24% zygotic 'Volkameriana' lemon seedlings using isoenzyme based markers. Hwang and Yeuh (1989) reported zygotic frequencies of 42.8% and 66.7% for 'Tankan' mandarin, using pollen from sour orange and *P. trifoliata*, respectively. Also, the authors reported a zygote frequency from 2.7% to 10.7% using 'Tankan' mandarin as the female parent and pollen from different orange cultivars. Hwang (1991) suggested that zygotic seedlings were identified by leaf morphology. He obtained 73% zygotic seedlings from 'Eureka' lemon crossed by trifoliate orange, and 38% to 56% zygotic seedlings from 'Eureka' and 'Lisbon' lemon cultivars crossed by sweet orange. Cristofani and Machado (1998) reported 6% zygotic seedlings of 'Cravo' lemon in a sample of 50 plants taken from a population of 576 produced in a greenhouse using RAPD markers. Bastianel *et al*. (1998) using RAPD, identified 26.7% hybrid seedlings from crossing 'Montenegrina' with 'King' tangerine species. For *C. volkameriana rootstock*, Garcia *et al*. (1999) reported only 22% zygotic seedlings from seeds derived from open pollination, and 60% of zygotic seedlings were obtained after pollination with *P. trifoliata*. Ruiz *et al*. (2000), using SSRs, classified 86.95% hybrid seedlings from crossing 'Flying Dragon' and 'Ortanique' tangor. Yun *et al*. (2007) obtained 0.0% to 13.4% hybrid seedlings from crossing female parents 'Miyagawa Wase', 'Okitsu Wase' and 'Shiranuhi' mandarins, and 'Ponkan' mandarin and 'Swingle' citrumelo male parents. Rao *et al*. (2008) crossed different mandarin and pummelo cultivars to obtain alternative rootstock towards sour orange and, using SSRs, identified 22.2% hybrid seedlings from these crosses. Garcia *et al*. (1999) suggested that zygotic embryos produced by self-pollination are less vigorous and may not be competitive with nucellar ones. The percentages of zygotic progenies in various citrus hybrids have been found to depend on the seed parent used (Spiegel-Roy *et al*., 1977), the pollen origin (Cameron and Soost, 1980; Soares-Filho *et al*., 1995), and environmental influences (Khan and Roose, 1988; Moore and Castle, 1988; Roose and Traugh, 1988).

In this study, using both 'Fremont' and 'Robinson' mandarin cultivars as the female parents produced the highest hybrid seedling ratio when 'Rio Red' cultivar was used as the male parent. The lowest hybrid seedling ratio was obtained when 'Midknight Valencia' pollen was used. When 'Fremont' and 'Robinson' mandarin cultivars were used as female parents with 'Rio Red' cultivar as the male parent, there was a significantly higher zygotic individual ratio in the F_1 population. When considering the whole F_1 population, the use of 'Midknight Valencia' as the male parent increased the nucellar seedling ratio to a greater extent than other orange male parents. Therefore, 'Mid-

knight Valencia' was not found to be useful for hybridization.

Tab. 2. Frequency of nucellar seedling obtained by controlled pollination with viable pollens from different orange and grapefruit cultivars in 'Fremont' and 'Robinson' mandarins

Female parents	Fremont			Robinson		
	No of seedling	Nucellar seedling		No of seedling	Nucellar seedling	
		No	Percent		No	Percent
Valencia Late	63	24	38.10	77	25	32.47
Rhode Red Valencia	85	32	37.65	79	28	35.44
Midknight Valencia	44	18	40.91	24	13	54.17
Rio Red	41	12	29.27	87	17	19.54
Total	233	86	36.91	267	83	31.09

The seedlings of each parental combination were assessed using SSR primers. Whereas some individuals were tested to be either either zygotic or nucellar using only one SSR primer, others were tested using more than one SSR marker. In combinations where the 'Fremont' mandarin was used as the female parent, the TAA41 primer was able to identify more zygotic individuals (47) than the other primers used (Tab. 3).

Also, in combinations where the 'Robinson' mandarin was used as the female parent, AG14 and TAA03 primers determined 59 and 58 zygotic individuals, respectively. In contrast, the CAT01, TAA01 and TAA45 primers (when 'Fremont' mandarin was used as female parent) and TAA45 and TAA52 (when 'Robinson' mandarin was used as female parent) were not useful in determining the identity of zygotic seedlings. When the whole F_1 population was considered, the AG14 and TAA03 primers were found to be more effective than other primers at distinguishing zygotic individuals, whilst the TAA45 primer was not able to identify zygotic seedlings. When a population is derived from a cross, all of the zygotic individuals show a genotype different from that of the mother at any discriminating locus, provided that the father has alleles different than those of the mother (Ruiz *et al*., 2000). Of the 24 seedlings coming from the combination of 'Robinson' mandarin as the female parent and 'Midknight Valencia' orange as male parent, five seedlings showed a banding pattern different from that of the mother plant when the TAA03 primer was used (Fig. 1).

Previous studies have indicated that the *Citrus* genus can be classified into three main groups as mandarin *(C. reticulata*), pummelo (*C. grandis*) and citron (*C. medica*). These studies made with isoenzyme (Asins *et al*., 1996), SSR marker (Luro *et al*., 2000) and sequence related amplified polymorphism (SRAP) marker (Uzun *et al*., 2009). The other citrus species such as lemon, orange, and grapefruit are derivatives from these three ancestral species (Nicolosi *et al*., 2000; Gulsen and Roose, 2001). The literature suggested that orange and mandarin were in the same group as shown in studies using isoenzyme (Novelli *et al*., 2000), SSR marker (Barkley *et al*., 2006) and AFLP marker (Pang *et al*., 2007).

Tab. 3. Number of zygotic individuals in several crossing populations tested using different SSR primers

Primer	Fremont				Robinson				Total
	Valencia Late	Rhode Red Valencia	Midknight Valencia	Rio Red	Valencia Late	Rhode Red Val.	Midknight Valencia	Rio Red	
AG14	3	-	9	19	20	-	-	39	90
ATC09	-	1	2	3	14	-	1	2	23
CAC33	11	21	-	4	2	9	2	21	70
CAGG9	6	-	-	2	-	-	-	12	20
CAT01	-	-	-	-	5	23	-	8	36
CAT02	19	-	5	9	-	-	4	21	58
TAA01	-	-	-	-	13	-	4	8	25
TAA03	6	23	-	-	27	22	5	4	87
TAA41	-	29	11	7	-	12	-	15	74
TAA45	-	-	-	-	-	-	-	-	-
TAA52	3	2	-	5	-	-	-	-	10

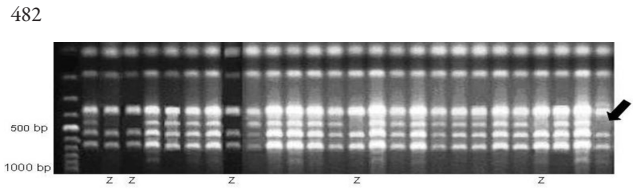

Fig. 1. Microsatellite TAA03 alleles banding pattern on 2% agarose gel Lane M: 100 bp molecular marker (Invitrogen); lanes 1 - 24 tested seedlings; lane R: 'Robinson' mandarin; lane MV: 'Midknight Valencia' orange; Z: zygotic genotypes

Results of this study agree with those of the previous studies. Female parents, 'Fremont' and 'Robinson' mandarins, and male parents of oranges had a genetic similarity range of 0.79-0.86 and 0.81-0.90, respectively, while these two mandarins had lower genetic similarity value, 0.72 and 0.73, respectively. Fang and Roose (1997) mentioned that mutations in most cases may cause significant changes in tree morphology, but with corresponding small modifications in the DNA sequence, which are therefore difficult to detect. Similarly, Luro *et al*. (2000) reported that oranges had an origin from a single parent and they are difficult to discriminate. Novelli *et al*. (2000) and Novelli *et al*. (2006) also reported that, despite considerable morphological differences, there is little RFLP, RAPD, and SSR polymorphism. Based on SSR markers, Cao *et al*. (2007) failed to detect differences among 16 *Satsuma mandarins* and, therefore, the possible mutation origin of these variants. Zygotic seedlings obtained from 'Fremont' and 'Robinson' mandarins as female parents and oranges as male parents had a similarity value of 0.86 and 0.95, respectively. The seedlings always have a higher similarity value with their maternal parents, which indicates decreasing genetic background thereby, increasing inbreeding which may cause lower tree performance under various environmental conditions (Fehr, 1993). Collectively, the results from this study suggest that the analysis of molecular markers may contribute significantly to citrus breeding programs.

Conclusions

This study demonstrated that the pollinators studied influenced the frequency of nucellar seedlings as detected by SSR markers. In the whole F_1 population, using grapefruit as the male parent resulted in more hybrid seedlings ratio than other orange male parents. The use of 'Midknight Valencia' as the male parent increased nucellar seedlings and was not found to be useful for hybridization. This result suggests that deliberate analysis of various combinations of parents is essential in citrus breeding programs. The use of SSR markers is an effective method to identify nucellar seedlings as being genetically identical to their maternal parent and may significantly contribute to breeding programs by decreasing the space and time needed to develop new cultivars.

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