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Identification of zygotic and nucellar tangerine seedlings (*Citrus* spp.) using RAPD

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ABSTRACT

The randomly amplified polymorphic DNA (RAPD) technique was used to distinguish nucellar and zygotic seedlings resulting from crosses between the Montenegrina (*Citrus deliciosa* Tenore) and King (*C. nobilis* Loureiro) tangerines. The aim of the present study was to develop tangerine varieties with a reduced number of seeds and organoleptic characteristics similar to the Montenegrina tangerine. Embryos were isolated from seeds, cultivated *in vitro*, and acclimated in a greenhouse. Four random primers were used to identify 54 plants of sexual origin from a total of 202 individuals. The degree of polymorphism of each primer was reflected in the number of zygotic plants obtained per primer. Cluster analysis of parents and progeny separated the individuals into distinct groups with a maximum genetic dissimilarity of 20%.

INTRODUCTION

The tangerine Montenegrina (*Citrus deliciosa* Tenore) is a highly acceptable fresh fruit because of its pleasant flavor. Its coloring, physical and chemical traits are enhanced by the climate and soil of Rio Grande do Sul, Brazil. These characteristics along with its late maturing season - it is ripe when similar fruits are scarce on the market - make it an excellent alternative crop, because of the better prices achieved. However, its large number of seeds has precluded export to Europe, which demands seedless fruits of good appearance and flavor. Crossing diploids (2x) of tetraploids (4x) to produce triploids (3x) has become a useful method for producing seedless citrus varieties (Soost and Cameron, 1985). One of the main problems found in citrus breeding programs is undesirable nucellar polyembryogenesis. Many polyembryonic tangerine varieties such as Montenegrina and King have been used in breeding programs. Consequently, the demand for methods to separate nucellar from zygotic embryos has increased.

Zygotic and nucellar embryos could be differentiated with the development of isozyme techniques (Torres *et al.*, 1978; Soost *et al*., 1980; Soost and Torres, 1981). Although there are about 18 known enzymatic systems for citrus (Ashari *et al.*, 1989), the accuracy of enzymatic analysis is influenced by the choice of enzyme and the type and age of the tissue analyzed (Roose, 1988; Asíns *et al.*, 1995). In preliminary isozyme tests, polymorphism was not found among the tangerines Montenegrina and King. Recently, molecular markers have been able to analyze DNA directly, without any influence from the environment or tissue age (Tansksley *et al*., 1989). Among these, random amplified polymorphic DNA markers (RAPD) have been widely used in citrus because of their assumed phenotypic neutrality and their ability to quickly and easily reveal a large number of markers. The technique has been used mainly for genotype typification, phylogenetic studies, mapping and mutant identification (Luro *et al.*, 1995; Cai *et al.*, 1994; Deng *et al.*, 1995). The RAPD technique does not need previous information about the targeted DNA and shows great polymorphism (Ferreira and Grattapaglia, 1995).

The objective of the present study was to identify Montenegrina and King tangerine hybrids using the RAPD technique. The results would be used to develop a breeding program for tangerine varieties with organoleptic characteristics similar to Montenegrina, eventually with fewer seeds.

MATERIAL AND METHODS

In vitro embryo culture

The parent plants used in this study came from the citrus collection at the Estação Experimental Agronômica da Universidade Federal do Rio Grande do Sul, Eldorado do Sul, RS. Controlled pollinations were carried out in September 1993 using Montenegrina tangerines as the female parent and King as the male.

The resulting seeds from the crosses were extracted and washed in running water to remove mucus. They were treated with 70% alcohol for 2 min, 2% sodium hypochlorite for 20 min and washed three times in sterilized water (Pescador, 1993). The embryos were removed with tweezers and a histological needle under a magnifying glass, placed in MS culture medium (Murashige and Skoog) and cultivated in a growth chamber for 45 to 60 days, until seedling formation was complete. These were placed on carbonized rice husks and turf substrate and acclimatized in a greenhouse.

Extraction of genomic DNA

Approximately 50 mg of leaves, dried in a drying oven for 24 h at 40^{o}C, was used for DNA extraction. Total DNA was extracted using Murray and Thompsons (1980) methodology and quantified by agarose gel electrophoresis according to Sambrook *et al.* (1989).

Amplification

Four out of 12 10-bp random primers revealed polymorphisms between the parents. These primers were OPI11 (5-ACATGCCGTG-3) OPH15 (5-AATGGCGCAG-3), P140 (5-AGGTCACTGA-3) and P141 (5-GGGGTTGACC-3).

Amplification reactions were prepared in a volume of 12.3 μl, containing 1.0 unit of Taq polymerase (Cembiot, RS, Brazil); 200 mM each of dATP, dTTP, dCTP and dGTP (Boehringer Mannheim); 1.3 μl of 10x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 2.0 mM $MgCl_2$ and 0.05% gelatine); 15 ng of primer; 15 ng genomic DNA, and 15 ml mineral oil was added to prevent evaporation. Amplification reactions were carried out in a MJ Research Inc. thermocycler programmed for 36 cycles of 1 min at 92°C, 1 min at 36°C, and 2 min at 72°C, followed by a final cycle of 10 min at 72°C.

The amplified and separated fragments were visualized in 1.4% agarose gel stained with 0.5 μg/ml ethidium bromide and photographed under ultraviolet light with Polaroid 667 film.

Data analysis

Presence (1) and absence (0) of intensely stained products were recorded and used in the analyses. Ten nucellar individuals, together with the zygotes and the parents (Montenegrina and King), were analyzed using numerical and multivariate analyses (NTSYS-PC) 1.7 Version (Rohlf, 1992). A similarity matrix was generated using SM (simple matching) coefficients and a dendogram constructed using the UPGMA method (unweighted pair group method).

RESULTS AND DISCUSSION

Controlled pollination of 30 Montenegrina flowers using King pollen resulted in eight fruits and a total of 80 seeds. Two hundred and seventy individuals were obtained from embryo culture of these seeds. Loss through contamination, physical damage or other causes was about 25%. Some studies have obtained up to 6.15 seedlings per seed (Machuka *et al.*, 1993), but the mean of the Montenegrina and King cross in this study was 3.37. This was probably associated with the degree of polyembryogenesis of the species used. Frost and Soost (1967) reported that some cultivars have numerous embryos per seed, whereas others have few extraneous embryos.

Figure 1 (A,B) shows RAPD amplification patterns obtained through comparisons of some F1 individuals with their parents using OPI11 primer (Figure 1A). Zygotic individuals (lines 69, 72a and 80) were identified by the absence of 2000-bp fragments, which are absent in the male parent King, but present in the female parent Montenegrina. Figure 1B shows RAPD patterns obtained with primer P141. F1 zygotes were identified by the absence of 1400-bp fragments (lines 24 and 26), which are present in the female parent, but absent in the male parent. Furthermore, 840-bp fragments (lines 21, 16 and 29), which are present in both parents, did not amplify.

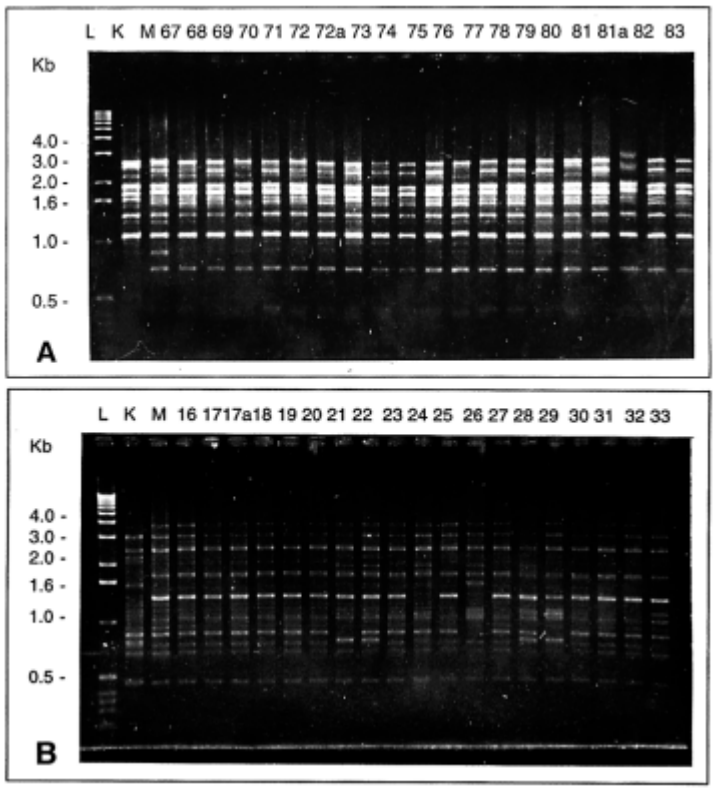

Figure 1 - Visualization of RAPD reaction of individuals from crosses of tangerines Montenegrina (*Citrus deliciosa)* and King (*C. nobilis*). A, Primer OPI11; B, Primer P141.

The primers used (OPI11, OPH15 , P141 and P140) amplified 31 well-resolved products, 12 of which were polymorphic. Different levels of polymorphism were obtained with each primer used. Primer OPI11 showed one polymorphic product, whereas three polymorphic products were obtained with primer P140, and four

polymorphic products with primers OPH15 and P141. These different polymorphism levels influenced the number of zygotic individuals identified by each primer, since the more polymorphic primers revealed a greater number of zygotic progeny (Table I). Thus, a wider selection of polymorphic primers may increase the chances of identifying zygotes, as different regions of amplification could be recognized in both parents and progeny.

Table I - Degree of polymorphism generated per primer and the number of zygotes identified among progeny of Montenegrina (*Citrus deliciosa* Tenore) and King (*C. nobilis* Loureiro) tangerines.

Primer	No. of polymorphic fragments	No. of identified zygotes*
OPI11	1	22
P140	3	27
OPH15	4	33
P141	4	38

*Some zygotes were identified by more than one primer.

Among the 202 plants, 54 F1 hybrids (26.7%) were identified with only four primers. Frost and Soost (1967) showed zygote frequencies of 78.7% and 14.02% for King and Willowleaf tangerines, respectively, using pollen from *Poncirus trifoliata.* Soost *et al.* (1980) found a zygote frequency of about 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 (Hirai *et al.*, 1986; Cameron and Soost, 1980; Roose and Traugh, 1988). The difference among the values obtained here and those in the literature may be attributed to pollination efficiency, loss of zygotic individuals during culture, and plant acclimatization. Environmental and genetic factors, in addition to nutritional and varietal factors, may also have affected zygote frequency (Khan and Roose, 1988; Roose and Traugh, 1988).

Data clustering (Figure 2) grouped hybrid and nucellar individuals and the parents. Only 10 nucellar individuals were presented, because all nucellar plants showed 100% similarity. Two large groups based on the markers were found. One group was formed by individuals similar to the male parent (King - K), and the other was formed by individuals similar to the female parent (Montenegrina - M). The latter was associated with a group of nucellar progeny. Maximum dissimilarity between hybrid individuals was 20%. Medina Filho *et al.* (1993) using isozymes showed the occurrence of twins and triplets in progeny from crosses involving different *Citrus* species and *P. trifoliata* with the following frequencies: monozygotic twins (1.5%), monozygotic triplets (0.15%) and dizygotic twins (0.315%). In the present study, the hybrids which make up the subgroups with 100% similarity came from different seeds. Thus, the possibility of twins was eliminated. According to the RAPD technique principle, trials with larger numbers of primers with these sub-groups could lead to differentiation of these individuals.

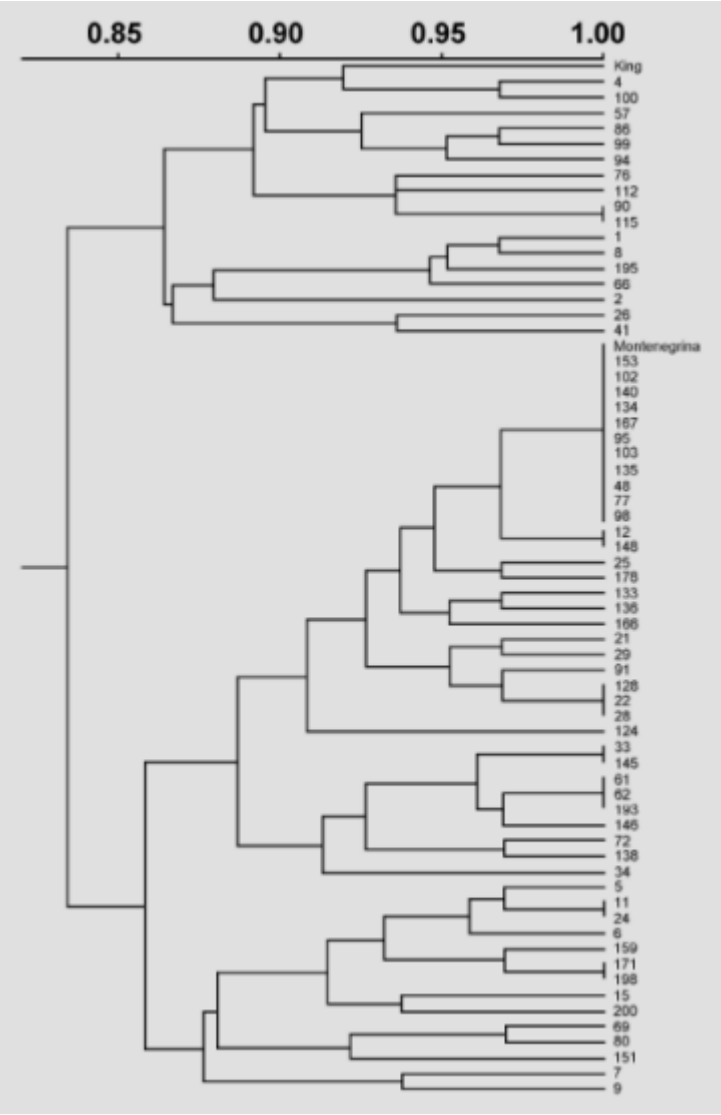

Figure 2 - Similarity among individuals from crosses of tangerines Montenegrina (*Citrus deliciosa*) and King (*C. nobilis*) generated by the SM (simple matching) coefficients using UPGMA grouping.

The RAPD technique was efficient in the identification of hybrids from crosses of Montenegrina and King, with the identification of 54 hybrids from a progeny of 202 individuals. Plants obtained through *in vitro* cultures of previously separated embryos probably increase survival and development of zygote embryos. This does not normally happen when the seed is placed to germinate directly in a substrate, which indirectly contributed to the zygote frequency obtained in this experiment.

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RESUMO

A técnica RAPD (random amplified polymorphic DNA) foi utilizada para distinguir plântulas nucelares e zigóticas resultantes do cruzamento entre as tangerineiras Montenegrina (*Citrus deliciosa* Tenore) e King (*C. nobilis* Loureiro). Este cruzamento foi realizado objetivando a obtenção de variedades de tangerineiras com características organolépticas de fruto semelhantes à tangerina Montenegrina e menor número de sementes. Embriões foram isolados das sementes e cultivados *in vitro* e aclimatizados em casa de vegetação. Utilizando-se de 4 primers de seqüência randômica foram identificadas 54 plantas de origem sexual de um total de 202 indivíduos. O grau de polimorfismo de cada primer refletiu no número de plantas zigóticas obtidas por primer, sendo o total de zigóticos identificados pela soma das informações geradas pelos 4 primers. Análise de agrupamento com os parentais e a progênie separou os indivíduos em grupos distintos com uma dissimilaridade genética máxima de 20%.

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