African Journal of Biotechnology Vol. 10(82), pp. 18965-18970, 19 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2810
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Identification of zygotic and nucellar seedlings in citrus interspecific crosses by inter simple sequence repeats (ISSR) markers

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Accepted 28 October, 2011

Interspecific sexual hybridizations were conducted between Yashar [(*Citrus changsha*) × (*C. paradisi* × *C. reticulata*)] as maternal parent and five different genotypes as parental including Page [(*C. clementina*) × (*C. paradisi* × *C. reticulata*)], Changsha (*C. changsha*), Ponkan (*C. reticulata*), Marrs (*C. sinensis*) and Hamlin (*C. sinensis*) in an effort to generate hybrid populations for scion breeding. A total of 227 plantlets were generated and screened to distinguish zygotic from nucellar seedlings. Parents and progenies were exposed to inter simple sequence repeats (ISSR) using different primers. Among 227 tested individuals, 67 hybrid and 160 nucellar seedlings were recognized. The results indicate that ISSR analyses are very efficient and reliable for identification of hybrids in polyembryonic citrus cultivars.

Key words: Citrus, fruit breeding, molecular markers, polyembryony, hybrid detection.

INTRODUCTION

Citrus is one of the most important fruit crops in the world. *Citrus* species are trees with persistent leaves, hesperidium fruits, and seeds without endosperm, and often with two or more nucellar embryos. Since nucellar embryos develop asexually by ordinary mitotic division of cells of the nucellus and no male gamete contributes to their formation, nucellar seedlings are identical to the seed parent (Frost, 1943).

In citrus breeding programs, sexual hybridization normally faces several reproductive impediments, such as apomixis and polyembryony that result in a cumbersome identification of hybrid seedlings (Frost and Soost, 1968). These characteristics are considered undesirable since the correct identification of hybrids is hindered by these conditions.

The Yashar [(*Citrus changsha*) × (*C. paradisi* × *C. reticulata*)] 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 Mazandarn province, Iran. These characteristics along with its late maturing season (it is ripe when similar fruits are scarce in the market) make it an excellent alternative crop, because of the better prices achieved. Despite these appropriate traits, generation of an early-ripening fruit cultivar could be valuable.

As aforementioned, one of the main problems found in citrus breeding programs, is undesirable nucellar polyembryogenesis. Many polyembryonic cultivars such as Page, Marrs, Ponkan, etc. have been utilized in Iran citrus breeding programs. Consequently, the demand for methods to distinguish nucellar from zygotic seedlings is highly required.

Identification of hybrids resulting from the cross between polyembryonic parents is extremely difficult when neither parent has a convenient dominant character. Scientists have developed different procedures, including morphologic (Hearn, 1977), chromatographic (Tatum et al., 1974), and isoenzymatic (Torres et al., 1982; Anderson et al., 1991) methods to

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Table 1. Primer sequences used in the study.

Primer code	Sequences (5′ – 3′)
N1	HVH (GA)7T
N4	(GA)8YG
N5	(AG)8YT
N6	(AG)8YC
N7	(AC)8YG
N8	(AC)8YA
N9	(AC)8YT
N10	(CA)8RG

recognize different hybrids.

However, none of these methods provide perfect confirmation to identity true nucellar seedlings (Ruiz et al., 2000; Tusa et al., 2002). Therefore, using the above methods frequently result in the exclusion of true hybrid seedlings from segregating populations.

Molecular markers are very useful tools to help plant breeders in facing this issue (Asins et al., 1998). For example in citrus, randomly amplified polymorphic DNA (RAPD) analysis has been used for distinguishing nucellar from zygotic seedlings (Bastianel et al., 1998; Rodriguez et al., 2004, 2005). Ruiz et al. (2000) described the use of simple sequence repeat (SSRs) markers as an alternative method to distinguish sexual from nucellar citrus seedlings. Oliveira et al. (2002) used SSRs to identify hybrids from primarily nucellar seedling populations. Rao et al. (2008) utilized RAPD and expressed sequence tag (EST)-SSR markers to characterize the zygotic and nucellar seedlings after introgression crosses of mandarin (*C. reticulata*) and pummel (*C. maxima*). Tusa et al. (2002) examined ISSR marker for identification of zygotic and nucellar seedlings in citrus interploid crosses.

The ISSR-PCR technique uses primers that are complementary to a single SSR and anchored at either the 5′ or 3′ end with a one- to three-base degenerate oligonucleotide (‘anchor’) (Zietkiewicz et al., 1994). This anchor ensures that the primer binds only to one end of a complementary SSR locus. The great number of amplicons generated consists of the region between neighboring and inverted SSRs. As a result, the highly complex banding pattern obtained will often differ greatly between genotypes of the same species.

In this study using ISSR markers, we were able to identify hybrids seedlings obtained from interspecific crosses between Yashar × Page [(*C. clementina*) × (*C. paradisi* × *C. reticulata*)], Changsha (*C. changsha*), Ponkan (*C. reticulata*), Marrs (*C. sinensis*) and Hamlin (*C. sinensis*) which are all polyembryonic.

MATERIALS AND METHODS

Crosses using Yashar (Y) as mother plant with Page (P), Changsha (Ch), Ponkan (Po), Marrs (M) and Hamlin (H) as pollen plants which all are early maturing season cultivars were carried out in Iran Citrus Research Institute, Ramsar, Iran. The resulting seeds from the crosses were extracted and washed in running water to remove mucus. Seeds were cultured into soil mixture (70% coco peat + 30% peat moss) in plastic pots, grown in a greenhouse under 12000-15000 lux light intensity and 25 to 27°C.

Within the framework of the above breeding program, scions of 227 derived putative hybrid plants were budded onto sour orange (*C. aurantium*) rootstock plants for further agronomic evaluation through field trials. Before performance of such expensive field trials which is time consuming and requires four to seven years after planting (Ruiz et al., 2000), the clear recognition of the pollen progenitor of presumed hybrid plants is needed.

DNA extraction

From each parent and progeny plant, four young leaves were taken and total genomic DNA was extracted according to Murray and Thompson (1980) with some modifications. The leaves were ground to a fine powder in liquid nitrogen and resuspended in cetyltrimethyl ammonium bromide (CTAB) extraction buffer (1% CTAB, 100 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.7 M NaCl, 2% sarcosyl and 140 mM 2-mercaptoethanol). The supernatant was extracted with chloroform–isoamyl alcohol (24:1), and precipitated in absolute ethanol and pellet resuspended in TE containing 10mg/ml RNAse. DNA concentration was measured spectrophotometrically (Nano Drop 1000) at 260 nm and diluted to 12.5 ng/µl.

PCR amplification

For DNA amplification, ten ISSR primers were initially tested and finally eight primers that produced polymorphic bands were selected for further analyses. The primer sequences were adopted from Fang and Roose (1997) (Table 1), and were synthesized by Cinnagen Co-Ltd (Iran). DNA amplification was carried out in 10 µl reactions containing 50 ng of template DNA, 0.2 mM total dNTPs, 0.5 µM primer, 1.0 µl of 10X PCR buffer, 1.5 mM of magnesium chloride and 1 unit of Taq polymerase (Cinnagen, Iran). Amplifications were performed under the following cycle program: initial denaturation step for 4 min at 94°C, followed by 36 cycles at 94°C for 30 s (denaturation), 50°C for 45 s (annealing) and 72°C for 120 s (extension), followed by a final extension step at 72°C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1X TBE (45 mM Tris-borate1 mM EDTA) and 0.5 µg/ml aqueous solution of ethidium bromide. About 10 µl of reaction products (with 2 µl of loading buffer 6X) were loaded and UV light.

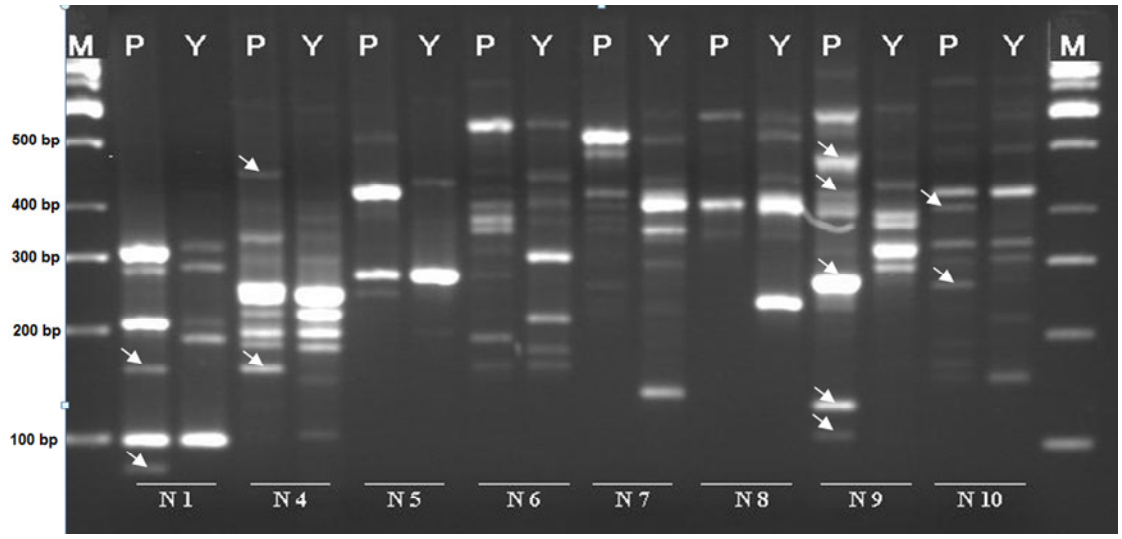

Figure 1. ISSR patterns of Page (P) and Yashar (Y) genotypes amplified with several ISSR primers for primer selection. The white arrowheads indicate the confirmed polymorphic markers N1, N4, N9 and N10, specific for pollen progenitor (Page). Other polymorphisms were observed that have not been confirmed (amplification by primers N5, N6, N7 and N8) and hence were not taken into consideration. (M) 100 bp ladder molecular marker (Fermentas).

Data analysis

Amplified bands from each primer were scored as present (1) or absent (0) for all the accessions studied. Only those bands showing consistent amplification were considered; smeared and weak bands were excluded from the analysis. Data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) software package version 2.02 (Rohlf, 2005). Cluster analysis was done by unweighted pair groups method arithmetic average (UPGMA) with Jaccard similar coefficient.

RESULTS AND DISCUSSION

Controlled pollinations of Yashar flowers using Page, Changsha, Ponkan, Marrs and Hamlin pollens resulted in a total of 179 seeds which resulted in 227 individual plants. 57 plants were obtained from the interspecific crosses of Yashar × Page, 49 from Yashar × Changsh, 52 from Yashar × Ponkan, 20 from Yashar × Marrs and 49 from Yashar × Hamlin. Average number of seedlings per seed was 6.15 (Machuka et al., 1993), 3.37 (Bastianel et al., 1998) seedlings per seed, but the mean of the crosses between Yashar and male parents in this study was 1.26. This was probably associated with the degree of polyembryogenesis of the species and/or type of medium used for seed germination. Frost and Soost (1967) reported that some cultivars have numerous embryos per seed, whereas others have few extraneous embryos.

Eight ISSR primers were selected to give polymorphic patterns between the parents and to analyze the zygotic offspring. Unique bands for each parent were expected to segregate in the offspring.

All of the ISSR primers were able to show from two to six polymorphic bands between the parents of each cross and these bands segregated in the progenies as expected (Figure 1). These different polymorphism levels influenced the number of zygotic individuals identified by each primer. 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 as was mentioned in previous study (Bastianel et al., 1998).

Figure 1 shows ISSR patterns of Y and P genotypes amplified with several ISSR primers to select primer for further analysis. The white arrowheads indicate the confirmed polymorphic markers N1, N4, N9 and N10, specific for pollen progenitor (Page). Other polymorphisms were observed that have not been confirmed (amplification by primers N5, N6, N7 and N8) and hence were not taken into consideration.

Figure 2 shows ISSR amplification patterns obtained through comparisons of some F1 individuals with their parents Y (as female) and P (as male) using N4 primer. Zygotic individuals (lines 6, 21, 22, 23 and 24) were identified by the presence of 260 bp fragments, which are present in Page, but absent in Yashar. Zygotic individuals (lines 13 and 14) were identified by the presence of 430 bp fragments, which are present in Page, but absent in Yashar. Zygotic individuals (lines 17, 18, 19 and 20) were identified by the absence of 300 bp fragments, which are present in Yashar, but absent in Page.

57 seedlings from the [Y] × [P] population were screened using N1, N4, N9 and N10 primers, and 35 of them were identified as nucellar seedlings. These primers were able to distinguish the six alleles present in the

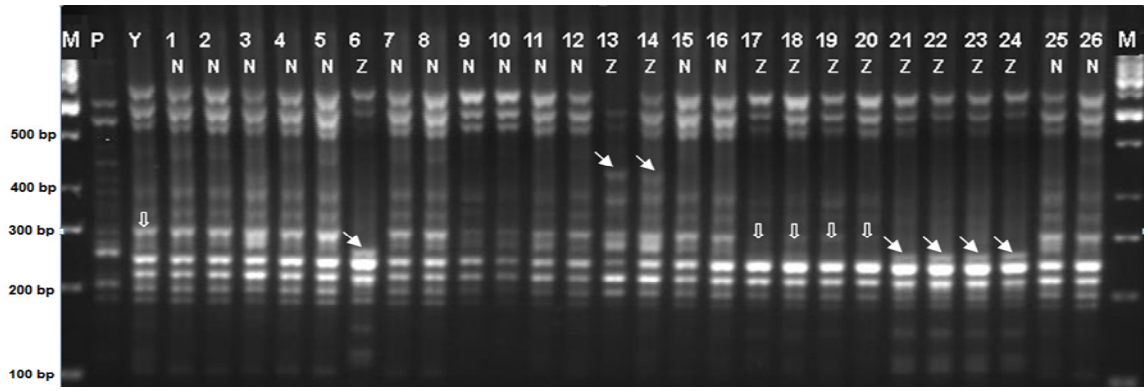

Figure 2. ISSR amplification patterns obtained through comparisons of some F1 individuals with their parents Page (P) as parental and Yashar (Y) as maternal using N4 primer. White arrowheads indicate zygotic individuals (lines 6, 21, 22, 23 and 24) that were identified by the presence of 260 bp fragments, which are present in Page, but absent in Yashar. Zygotic individuals (lines 13 and 14) were identified by the presence of 430 bp fragment, which are present in Page, but absent in Yashar. Zygotic individuals (lines 17, 18, 19 and 20) were identified by the absence of 300 bp fragments (hollow arrowheads), which are present in the Yashar, but absent in Page. (M) 100 bp ladder molecular marker (Fermentas).

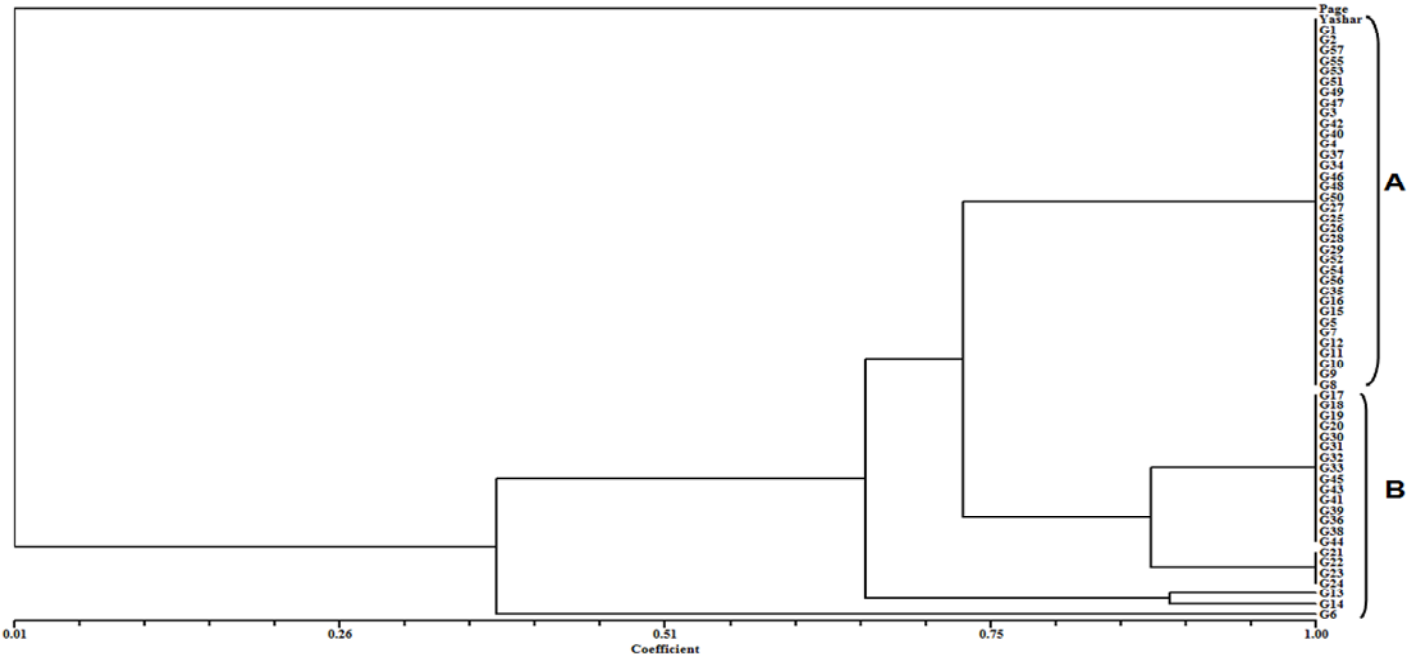

Figure 3. Dendrogram analysis by UPGMA method with jaccard similarity coefficient among 57 progenies from crosses of Yashar and Page. G = individual.

population; therefore the genotype of every plant could be unmistakably assessed.

Considering the dendrogram (Figure 3), hybrid and nucellar plants obtained from cross between Yashar and Page which identified by ISSR markers, and were clustered into two major groups (A and B). Group A contained 35 nucellar individuals, because all nucellar plants showed 100% similarity with female parent (Yashar). Group B was formed by plants similar to the male parent (Page). Maximum dissimilarity between hybrid plants was 60%.

The genotypes of the seedlings resulting from the cross between [Y] × [Ch] were identified using N1, N4 and N6 markers. Out of the 49 tested progenies, 39 were nucellar individuals.

ISSR alleles amplified from [Y] × [Po] and their progenies using N4, N5 and N8 primers showed that 37 out of 52 seedlings were nucellar plants.

Three of the zygotic seedlings resulted from the cross between [Y] × [M]; were identified through genetic analysis of the N4 and N7 primers.

Among the 49 tested plantlets from [Y] × [H], primers N1, N4 and N9 were able to detect 32 nucellar genotypes.

In this experiment, among the 227 plantlets, 67 individuals (29.5%) were identified as hybrid using aforementioned primers. Frost and Soost (1967) found zygote frequencies of 78.7 and 14.02% for King and Willowleaf tangerines, respectively, using pollen from *Poncirus trifoliata*. Cameron and Soost (1980) found a zygote frequency of about 85% using King as the female parent and pollen from Parson Special tangerine. Bastianel et al. (1998) demonstrated zygote frequency of 26.7% for Montenegrina tangerine using pollen from King tangerine. Rodriguez (2004) reported that in *Citrus volkameriana*, 26% of seedlings from polyembryonic seeds are classified as zygotic. Some studies revealed 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 figures obtained here and those in the literatures may be attributed to pollination efficiency and plant acclimatization. Environmental and genetic factors, in addition to nutritional and varietal factors, may also have affected zygote frequency (Kepiro and Roose, 2007).

Conclusion

After hybridization between Yashar as maternal and Page, Changsha, Ponkan and Hamlin as paternal parents with the aim of producing new cultivars, a population including 227 individual seedlings were generated and subjected to screening experiments by ISSR markers, in order to distinguish zygotic from nucellar seedlings. Among the tested plantlets, 67 hybrids and 160 nucellars were recognized.

In addition to many advantages of ISSR marker, this study has confirmed the potential of ISSR markers for plant genetics study in *Citrus*, specially identification of zygotic individuals from nucellar plants. As already cited by other authors, the ISSR advantages reside mainly in their capability to simultaneously detect many loci in a simple and cost-effective manner, requiring no previous knowledge of the genome sequence, as in the case of other methods such as SSR. It requires only 10 to 30 ng of DNA template, allowing a very early screening of the progeny (Zietkiewics et al., 1994; Goodwin et al., 1997). These features are extremely useful when a mixed hybrid population has to be quickly analyzed and characterized. Furthermore, the ISSR technique uses highly specific 18 bp long primers, guaranteeing higher reliability and repeatability than other techniques, such as RAPD (Bornet and Branchard, 2001).

ACKNOWLEDGEMENT

The authors thank the Citrus Research Institute, Ramsar, Iran for their support.

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