



DISTINGUISHING OF ZYGOTIC AND NUCELLAR SEEDLINGS IN CITRUS ROOTSTOCKS USING ISSR TECHNIQUE

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ABSTRACT

The objective of this study is to evaluate the morpho-based method (common system) which used to distinguish between zygotic seedling and nucellar seedling of Cleopatra mandarin and Sour orange rootstocks using ISSR technique. Eight ISSR primers were used to check true-to-type of three mother-plants and nine seedlings of two citrus species, Cleopatra mandarin from Giza nursery and Sour orange from Benha nursery. Results showed that ISSR markers were a utility to identify nucellar or zygotic seedlings and check seedlings true to the type of mother plant. Three Cleopatra mandarin seedlings (33.3%) were identified as nucellar seedlings and six seedlings (66.7%) were identified as zygotic seedlings when using ISSR5 primer. While this percentages were different when using ISSR5 primer with Sour orange seedlings, which revealed one of them (11.1%) was identified as a nucellar seedling and eight seedlings (88.8%) were identified as zygotic seedlings. This suggested that the common method used until now for selection the nucellar seedlings in nursery is not perfect. Finally, that screening of seedlings for early identification of putative zygotic seedlings by ISSR analysis for random and various combinations is essential in citrus to maintain the genetic characteristics of the citrus rootstocks in orchards.

Key words: Genetic variability, ISSR markers, citrus rootstocks, zygotic seedlings, nucellar seedlings.

INTRODUCTION

Citrus is a very important economic crop in Egypt with a production of 4,646,579 metric tons (Mt) on 1,070,478 hectares according to the **Yearly of Statistic and Agricultural Economic Dept. (2015)**. The citrus cultivated depend on the rootstocks used which play a key role for better growth and production (**Garcia-Sanchez et al., 2003**). In Egypt, Sour orange is using extensively, and recently Volkamer lemon is more widely used in sandy soils regions (**Salem and Sheta, 2002**). These rootstocks are produced by different methods but the optimum methods by seeds. But seeds of many important citrus cultivars produce zygotic embryos as well as nucellar embryony which consider as

unique botanical feature (**Frost and Soost, 1968**). Nucellus cells grow much more vigorously than the zygotic embryos and develop growers genetically identical to mother plant (**Xu et al., 2013**).

This botanical feature due to variation between citrus species trees in orchards in resistance unfavorable factors such as abiotic factors: drought, flooding, salinity, mineral deficiency and toxicity, metal toxicity, heat, cold, soil temperature, pH, etc., and biotic factors such as, plant diseases, insects and nematodes (**Louws et al., 2010; Ghrab et al., 2014; Castle et al., 2016**). So, the differentiation between seedlings develops by zygotic embryos or nucellar embryony as early in nursery is the very important agricultural practices before

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transferring these seedlings to the orchards. But up to now, the farmer finds difficulty to distinguishing zygotic from nucellar seedling.

Now a days, the morphological method still a common practice in nursery until now to distinguish zygotic from nucellar seedlings. However, it is very difficult to identify both the nucellar and zygotic seedlings (**Kumar and Rani, 2013**). The plant breeder keeps on the zygotic plantlets and remove the nucellar ones produced from the same seed by some morpho-traits such as: the zygotic plantlets were generally weak than nucellar plantlets, but it's strongest in growing compared with nucellar embryos when the zygotic embryos are still survival (**El-Hamady *et al.*, 2009**). Hence, this practice is effective in removing some zygotic plantlets, but not all (**Ruiz *et al.*, 2000**).

Fortunately, the molecular markers are considered important tools to detect higher level of polymorphism between samples. Among these markers the inter-simple sequence repeats (ISSR) that is effective to study genetic diversity between different citrus rootstocks species in many citrus genetic studies (**Fang and Roose, 1997; Liang *et al.*, 2007; Shahsavari *et al.*, 2007**). So, the aim of present study is to evaluate the morpho-based method (common system) which used to distinguish between zygotic seedling from nucellar seedling of Cleopatra mandarin and Sour orange using ISSR technique.

MATERIALS AND METHODS

DNA Extraction

Nine samples from seedlings and three mother-plants of two citrus species; Cleopatra mandarin from Giza nursery and Sour orange from Benha nursery, Egypt were used. DNA was obtained from young healthy leaves for mother-trees and seedlings by grinding them with 750µl of preheated (65°C) extraction buffer which contained

2% hexadecyltrimethylammonium bromide [CTAB], 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0. Mixtures were incubated at 60°C for 30 minutes, then extraction procedure was completed with Genomic Plant Kit (Zymo scientific Inc., USA) by following the manufacturer's instructions. The DNA quality checked with electrophoresis by agarose gel 1% (*W/V*). After quality check, DNA was taken to perform genetic analysis.

ISSR Analysis

A 25 ml of PCR reaction mixture contained a 0.25 unit of *Taq* DNA polymerase; 5 µl PCR buffer 1x; 2.5 µl primer and DNA sample (200 ng) to each sample tube. The reaction was performed in a PCR thermocycler with 35 cycles, each consisting of 1 min at 94°C, 1 min at annealing temperature (40°C), and an extension step of 2 min at 72°C. After 35 cycles, a final step of 7 min at 72°C was allowing to complete the synthesis of DNA strands. Eight ISSR primers were used (Table 1). Amplification products were analyzed by electrophoresis on 1.5% agarose gels, buffered with 1x TAE and stained with ethidium bromide (EtBr). The fragments were visualized using ultraviolet (UV) light source. Fragments were detected using TotalLab-120 software (Nonlinear Dynamics, USA) according to software manual. fragments were scored as 1 (present) or 0 (absent). Then, the principal coordinate analysis (PCoA) was carried out.

RESULTS AND DISCUSSION

Three mother-trees (P₁, P₂, and P₃) and nine seedlings (S₁, S₂, S₃, S₄, S₅, S₆, S₇, S₈ and S₉) of two citrus species, Cleopatra mandarin from Giza nursery and Sour orange from Benha nursery were screened.

Results showed that RAMP-GAC and ISSR5 primers were selected among all ISSR primers according to their polymorphic patterns between the Cleopatra mandarin

Table (1): List of ISSR primers used in this study

No.	Primer	Primer sequence (5' 3')
1	ISSR11	5' [AG] ₈ GT 3'
2	ISSR7	5' [GA] ₈ T 3'
3	LK7	5' CCA [CT] ₈ 3'
4	RAMP-GAC	5' G [AC] ₉ 3'
5	ISSR5	5' [AG] ₈ T 3'
6	RAMP-TAG	5' T[AG] ₉ 3'
7	ISSR14	5' [CA] ₈ GG 3'
8	ISSR8	5' [CT] ₈ G 3'

mother-trees and their seedlings (Fig. 1). Gel pattern of RAMP-GAC primer (Fig. 1-A) observed that three seedlings (S₂, S₄ and S₇) were identified as zygotic seedlings by the absent of three fragments from S₂ individual (577, 631 and 697 bp), four fragments from S₄ individual (631, 697, 852 and 1023 bp) and three fragments from S₇ individual (631, 697 and 771 bp), which was presented in each of mother-trees and the other six seedlings. In addition, ISSR5 primer detected six seedlings (S₄, S₅, S₆, S₇, S₈ and S₉) as zygotic seedlings by the presence of 1449 bp fragment which was absent in mother-trees and the other three seedlings (Fig. 1-B). Also, no difference was found among each of the mother-trees with the both primers.

The principal coordinate analysis (PCoA) result divided the 12 individuals into three clusters (Fig. 1-C). The three mother-trees (P₁, P₂ and P₃) were grouped together in cluster I with two seedlings (S₁ and S₃). But S₂ and S₇ seedlings were tended to cluster together into cluster II. While the five remaining seedlings (S₄, S₅, S₆, S₈ and S₉) were clustered into cluster III. This PCoA graph indicted that S₁ and S₃ seedlings had the same genetic structure of the mother-trees which could be produced from nuclear

embryo. In contrast, the other seedlings into the two different clusters were attest the variability of their genetic background compared with their mother-trees which could be produced from zygotic embryo or pollinated from another citrus species.

On the other hand, ISSR5 primer detected one nucellar seedling of Sour orange (S₁) compared with two mother-trees (P₁ and P₂) pattern (Fig. 2-A). But P₃ mother-plant have one additional fragment (353 bp) not showed with other mother-plants, that make P₃ mother plant is a different genetic structure. And this was confirmed with RAMP-TAG primer by not having two fragments (970 and 1096 bp) in its pattern (Fig. 2-B). But RAMP-TAG primer identified five seedlings only (S₂, S₃, S₄, S₆ and S₇) as zygotic seedlings because the two fragments (970 and 1096 bp) don't show in its pattern.

This genetic diversity was shown in the principal coordinate analysis (PCoA) resulting. The 12 individuals of sour orange were clustered into five clusters (Fig. 2-C). The two mother-trees (P₁ and P₂) grouped together in the same cluster with three seedlings (S₁, S₅ and S₈). While each of P₃ mother-trees and S₆ and S₉ seedlings separated into three clusters. But S₂, S₃, S₄

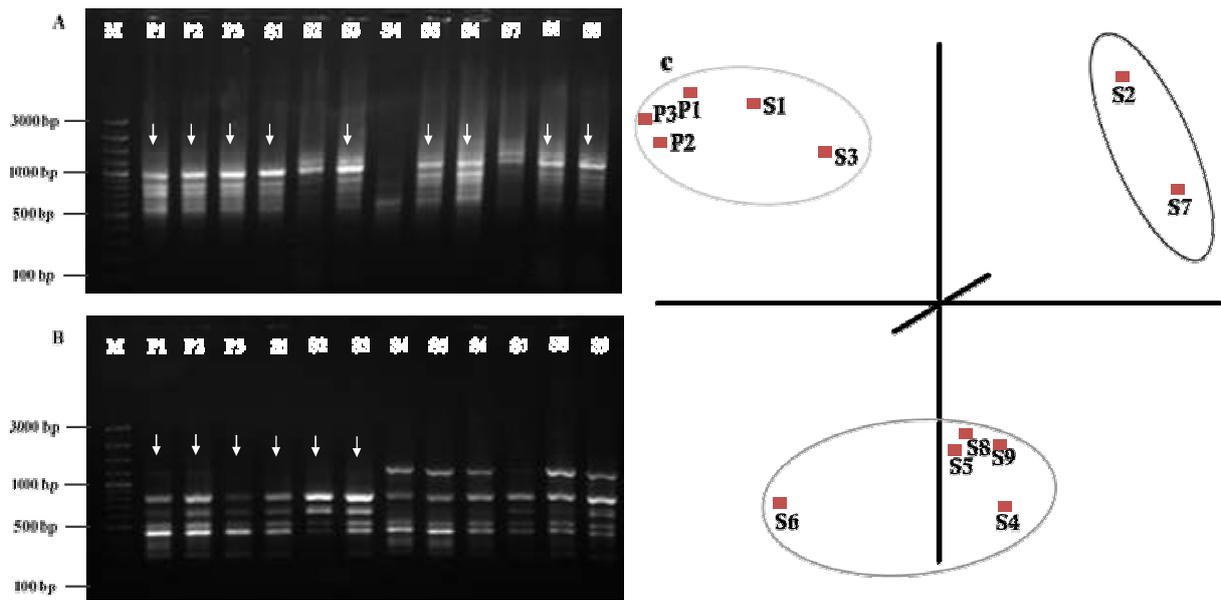


Fig. (1): ISSR amplification patterns to three Cleopatra mandarin mother-trees and their nine seedlings. (P), (S) and (M) refer to mother-plants, seedlings, and 100 bp ladder molecular marker, respectively.

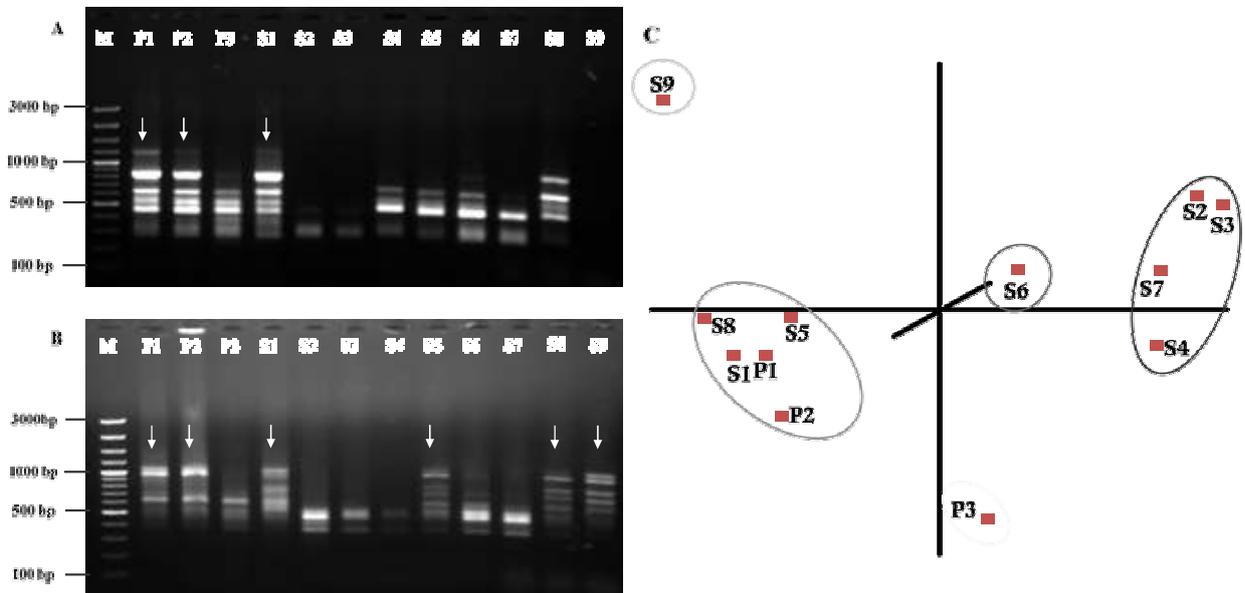


Fig. (2): ISSR amplification patterns to three Sour orange mother-trees and their nine seedlings. (P), (S) and (M) refer to mother-plants, seedlings, and 100 bp ladder molecular marker, respectively.

and S₇ tend to cluster together into cluster V. Depending upon this PCoA results, it's certainly found the P₁ and P₂ mother-trees with S₁, S₅ and S₈ individuals attest that these seedlings have the same genetic structure and produced from nuclear embryo. On the other hand, separate the other parent (P₃) and five seedlings into four different clusters refer to the genetic variability was differed from the mother-trees which could be produced from zygotic embryo or pollinated from another citrus species.

In brief, these results noticed that three Cleopatra mandarin seedlings (33.3%) were identified as nucellar seedlings and six seedlings (66.7%) as zygotic seedlings using RAMP-GAC primer. While this percentages were different with using ISSR5 primer which identified three seedlings (33.3%) as nucellar seedlings and six seedlings (66.7%) as zygotic seedlings. But, Sour orange seedlings revealed one of them (11.1%) as nucellar seedling and eight seedlings (88.8%) as zygotic seedlings using ISSR5 primer. While this percentages were changed when using RAMP-TAG primer which identified three seedlings (33.3%) as nucellar seedlings and six seedlings (66.7%) as zygotic seedlings. This finding agree with **Kashyap et al. (2018)** who recognized 37 (53.6%) as zygotic and 32 (46.3%) as nucellar Khasi mandarin (*Citrus reticulata* Blanco) seedlings among 69 tested individuals, using four ISSR and three RAPD primers. **Xiang and Roose (1988)** reported that in Taiwanica sour orange, greater than a 30% of seedlings from polyembryonic seeds were classified as zygotic seeds. Consequently, ISSR markers are a utility to identify nucellar or zygotic seedlings and check seedlings true-to-type of mother plant.

These results revealed that the embryo type which developed seedlings were variable in the same nursery, some of seedlings were divided from a nucellar

embryo and the other from a zygotic embryo. These lead to a difference in genetic structure among the same species seedlings in nurseries. It is known that the seedling developed from a nucellar embryo is genetically similar to the parent seed, whilst a zygotic embryo is different from parent seed (**Ollitrault and Navarro 2012; Khan et al., 2017**) This consider as major problem to citrus growers due to a different orchard in horticultural performance, e.g. growth and characteristics of fruit (**Cordeiro et al., 2006; Rao et al., 2008**). Finally, this result suggested that screening of seedlings for early identification of putative zygotic seedlings by ISSR analysis of random and various combinations of seedlings is essential in citrus to guarantee the origin of these seedlings. ISSR5 primer is a strong tool to distinguish between zygotic seedling from nucellar seedling of Cleopatra mandarin and Sour orange.

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تمييز الشتلات الجنسية والنيوسيلية في أصول الموالح باستخدام واسمات التتابعات البيئية البسيطة المتكررة

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يهدف هذا العمل إلى تقييم استخدام الطريقة المورفولوجية (الطريقة الشائعة) للتمييز بين الشتلات الجنسية والنيوسيلية لكل من الكليوباترا وال نارنج باستخدام تقنية واسمات التتابعات البيئية البسيطة المتكررة، وقد استُخدم لذلك ثمان بوادي للتأكد من مطابقة النوع لتسع شتلات من نوعي للموالمح الكليوباترا من مشتل الجيزة، وال نارنج من مشتل بنها لثلاث أشجار أمهات، وأظهرت النتائج أن بوادي واسمات التتابعات البيئية البسيطة المتكررة لها القدرة على التعرف على الشتلات الجنسية أو النيوسيلية والتأكد من صحة مطابقتها لنوع أشجار الأمهات، وكشف البادي ISSR5 عن أن ثلاث شتلات من الكليوباترا كانت نيوسيلية (٣٣,٣%) وكانت ست شتلات جنسية (٦٦,٧%)، بينما اختلفت تلك النسبة باستخدام البادي ISSR5 مع شتلات النارنج، فأظهر أن واحدًا من تلك الشتلات كان نيوسيلي (١١,١%) وكان ثمان شتلات جنسية (٨٨,٨%)، وذلك يدل على أن الطريقة التقليدية المستخدمة حتى الآن لاختيار الشتلات النيوسيلية في المشاتل غير مثالية، وفي النهاية يمكن اقتراح أنه من الضروري عمل مسح مبكر للشتلات للتعرف على الشتلات الجنسية المحتملة باستخدام واسمات التتابعات البيئية البسيطة المتكررة لمجموعة عشوائية ومختلفة من الشتلات للحفاظ على الصفات الوراثية المتماثلة لأصول الموالمح بالبستان.

الكلمات الإسترشادية: التباين الوراثي، واسمات التتابعات البيئية البسيطة المتكررة، أصول الموالمح، الشتلات الجنسية، الشتلات النيوسيلية.

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