International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol5, Issue1, 2014, pp 25-30
http://www.bipublication.com

# IDENTIFICATION OF ZYGOTIC AND NUCELLAR SEEDLINGS OF *Citrus reticulata* AND *Citrus aurantifolia* using RAPD

**B. Mondal[1] and R Saha[2]**

[1]BCKV, Directorate of Agriculture, Kalyani, Nadia, West Bengal
[2]Agriculture Department Govt. Of West Bengal
* Corresponding author. bidishabm3@gmail.com, Tel: 91-9433164162

[Received-21/11/2013, Accepted-20/02/2014]

**ABSTRACT:**

Citrus is a very important fruit crop of India with a very special trait called polyembryony. This trait could be effectively utilized for citrus propagation in nursery or hybridization work. The proper identification of zygotic and nucellar seedlings of *Citrus reticulata* and *Citrus aurantifolia* may lead to proper management of the elite germplasms in mass scale propagation. Conventional techniques of 'Off type' rouging and other morphological identification techniques are not full proof. Molecular marker may be used for discrimination of different seedlings. Cheap and cost effective RAPD technique is used for separation of zygotic and nucellar seedlings of a single seed of both species with 20 non specific decamer primers. Primer OPN12, OPA18, OPD08, OPA13 OPA07, OPM06 gave polymorphism in *C. reticulata*. OPH15, OPAT04 differentiates seedling in *C. aurantifolia*. All total 14 amplicons were identified with these 8 primers those are effective in zygotic and nucellar discrimination. Sequencing of the amplicons could generate nucleotide sequence associated with the trait. Polyembryony character is regulated by several environmental factors hence specific marker selection for specific eco region is important. The universal primers identified here are important for detection of polyembryony trait of both *C. reticulata* and *C. aurantifolia* species of this subcontinent.

**Key words**: *Citrus reticulata*, *Citrus aurantifolia*, RAPD, zygotic, nucellar, amplicons

## [1] INTRODUCTION:

*Citrus* polyembryony can be used to propagate plants through nucellar embryos. Both *Citrus reticulata* and *Citrus aurantifolia* shows polyembryony. Nucellar apomixes forms multiple nucellar embryos along with a fully developed zygotic embryo. The percentage of nucellar embryos varies from species to species. The nucellar embryos were somatic in origin and forms true to the type progeny [1]. Nucellar embryos could be utilized for preservation of the desirable heterozygous state of mother plant i.e fixing heterosis. Citrus germplasms can be introduced as budwood or seed. Budwood ensures trueness to type but increases the risk of exotic diseases and pathogen infestation. The risk of introducing diseases is less with seeds, because no citrus disease has been definitely proven to be seed transmissible [2]. Citrus shows long juvenility and exploitation of morphological marker for hybrid identification is difficult. Enzymatic darkening, gas chromatography, isozyme analysis had been used to study the difference of zygotic and nucellar ones. The product of gene expression may reflect some environmental influence and produce erratic results.

Among the recently used molecular markers Random Amplified Polymorphic DNA marker is simple, cost effective and reliable. RAPD marker is used for a long time by researchers all over the world for its simplicity. SSR markers are co-dominant and more reliable but are not so useful for intraspecific studies and also expensive for regular use by small growers and farmers of North Eastern Hilly region. RAPDs have been extensively used in assessing relationship amongst various Citrus accessions [3], genotype identification [4], estimation of relationship [5] and zygotic and nucellar detection [6]. Moreover, in citrus several traits of horticultural importance, including resistance to citrus tristeza virus and nematode resistance [7] and dwarfing [8] have been tagged with RAPD markers. Most of the RAPD markers could be converted into reliable sequence specific PCR based markers or sequence characterized amplified regions (SCARs) [9], [10]. The objective of this work is to screen some universal decamer primers those can differentiate hybrid and somatic seedlings of *Citrus reticulata* and *Citrus aurantifolia*.

## [II] MATERIALS & METHOD:

One (*Citrus reticulata)* plant from Mirik, Darjeeling and another *Citrus aurantifolia* plant of Nadia, West Bengal, India were marked for high productivity, regular bearing and fruit quality. Mature fruits were collected and brought to the laboratory. Seeds collected from five representative fruits of each plant were surface sterilized with 0.1% mercuric chloride solution, placed between two layers of moist sterile cotton pad in Petri dishes, and incubated for 5 to 7 days at 35°C to germinate. Upon swelling of seeds, the germinating nucellar and zygotic embryos were identified following the procedure standardized by [11]. Under aseptic conditions, the integument of the mature seed was carefully rolled away by making a longitudinal incision with a fine scalpel from the micropylar end. The germinating embryo holding the two original cotyledons and originating from the micropylar end was considered as the zygotic embryo. All other germinating embryos under the integument, each with two newly differentiating tiny cotyledons, were taken as nucellar embryos [11], [12]. The number of embryos and seedlings originating from the zygotes and from nucellar tissue were recorded. More than one seedling developing from a single point by the fission of the original zygotic or nucellar embryo were considered as twins. After observation for primary records, the germinating seeds were allowed to grow in aseptic conditions on a cotton bed for another 10 to 12 days, and then put into a sterile soil-sand-organic matter mixture (2:1:1) under controlled conditions with high humidity for further growth of the seedlings, and were marked separately according to their origin. The growth pattern of different seedlings were carefully noted and recorded. When the seedlings were of seven month old with sufficient leaves for DNA extraction was available four plants with good growth were selected. One zygotic and another nucellar seedling from each species were selected.

### 2.1 DNA extraction and RAPD analysis:

Variability in banding pattern among the nucellar and zygotic seedlings developed from a single seed identified by the procedure mentioned above were investigated by RAPD analysis. RAPD analysis was carried with 20 Operon decamer primers selected by preliminary screening to give polymorphism and reproducible fragment patterns in both the species [13]. Genomic DNA was extracted from the soft leaves of the seedlings using the Plant DNA CTAB Extraction Kit (KT-55) of Merck-Genei (Bangalore, India). The quantity and amount of DNA were determined as described by [14]. Amplification was achieved by the protocol outlined by [15], with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10´ buffer, and 15 ng of decamer primers (Eurofins) in a total volume of 25 µL. The amplification was performed in a thermocycler (Gene Amp PCR System 9700, Applied BioSystems). Total reaction consisted

of 45 cycles, each cycle comprising three steps (denaturation at 92°C for 30 seconds; annealing at 38°C for 30 seconds; extension at 72°C for 1 minute), with an initial denaturation at 94°C for 30 seconds and a final extension at 72°C for 5 minutes, followed by cooling at 4°C. Amplification fragments were separated on 1.5% agarose (Merck-Genei) gels containing ethidium bromide (0.5 µg per mL of agarose) at 60 V for 6 hours in Tris Borate EDTA buffer. The gel was visualized and photographed under UV excitation using an electronic dual wave transilluminator system (Ultra.Lum Inc., USA). Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). The size of the fragments (molecular weight in base pairs) was estimated by using a 100-bp ladder marker (Merck-Genei), which was run along with the amplified products. The primers that could generate differential banding patterns of the seedlings of different origins (nucellar and zygotic) of a seed were noted.

**[III] RESULTS:**

Occurrence of more than one embryo within a matured seed was a common phenomenon in both *Citrus reticulata* and *Citrus aurantifolia*. 100 seeds from each species were analysed to count and differentiate multiple embryos on the basis of morphology and spatial position. Three to five days after incubation in the moist chamber, when the seeds became turgid and swollen, the embryos were distinctly visible on removal of the integument. The zygotic embryo was present at the micropylar region holding the two original cotyledons of the seed. This embryo was usually larger than the other embryos and also took the most space in a seed. In most cases, rudimentary hypocotyls of the zygotic embryos became visible after removal of the inner seed coat. Embryos originating from the nucellar tissues were tiny, green and heart-shaped, and were crowded at the micropylar region, or sometimes over the cotyledons of the zygotic embryos. Most of these could be easily separated when the integument was rolled away from the nucellus during the dissection of the seed. Individual nucellar embryos had two cotyledons, and rudimentary radicals and plumules were present in between the two cotyledons. The cotyledons covered the greatest area of an embryo.

In addition to the presence of the single normal zygotic embryo, twin and triplet zygotic embryos were also observed in a large number of seeds. During the further course of development, these embryos formed twin or triplet zygotic seedlings [16]. The seedlings originating from zygotic and nucellar embryos of *Citrus reticulata* and *Citrus aurantifolia* shows zygotic: nucellar ratio of 1:3 and 1:2. The seedlings developed from the only zygotic embryo, and full grown matured nucellar embryos of the same seed of both *Citrus reticulata* and *Citrus aurantifolia* were grown in separate pots with addition of organic matter and coir pith. The DNA extracted from those four plants when run with 20 selected RAPD primers gave some interesting results.

Six primers were able to differentiate nucellar and zygotic in *Citrus reticulata* while two primers proved useful in *Citrus aurantifolia*. All total 36 bands were generated in *C. reticulata* and 46 in *C. aurantifolia*. Some of the primers were not able to generate bands in *C. aurantifolia* zygotic seedlings which require further confirmation. Primer OPN12, OPA18, OPD08, OPA13, OPA07, OPM06 gave polymorphism in C. reticulata. OPH15, OPAT04 differentiates seedling in C. aurantifolia. 14 amplicons were found (table II) with ability to differentiate zygotic and nucellar plants. If these amplicons retain their stability in repeated studies then by sequencing of the amplicons precise markers could be generated.

**[IV] DISCUSSION:**

The detection of zygotic and nucellar seedlings by molecular profiling would be easier where there was a confirmed out-crossing from genetically different but known pollen sources[17]. Also zygotic seedlings resulting from self pollination could be distinguished

from nucellars if they are homozygous at one or more loci in which the seed parent was heterozygous [18]. In the present RAPD analysis, the detection of zygotic and nucellar seedlings was made within the natural seed-propagated population without knowledge of sources of pollen parents or any control over them. Eight RAPD markers were able to differentiate the seedlings according to their developmental origin. This easy procedure for the detection of nucellar and zygotic seedlings of a population using RAPD markers should be further confirmed with a controlled experiment with a controlled pollination system.

Polyembryony is controlled by type of pollinator, viability of the pollinator, plant nutrition, air temperature, soil humidity, wind velocity etc [19]. Factors affecting pollination, fertilization and seed development also affects polyembryony. Due to the impact of such environmental factors polyembryony in each country may vary [20]. So for different ecological region polyembryony may be affected [21]. The eight decamer primers were able to differentiate nucellar and zygotic seedlings of *Citrus reticulata* and *Citrus aurantium* of Indian subcontinent.

## [V] CONCLUSION:

Polyembryony varies widely from species to species with ecological effect. So, it is important to develop region specific markers. In India this kind of research is rare. The finding of some universal primers and RAPD amplicons associated with polyembryony is a unique finding. Refined work may lead to the construction of SCAR or CAPS and distinct primers for this important polyembryonic trait of Citrus.

## ACKNOWLEDGEMMENT:

The authors sincerely acknowledge the financial assistance provided by DBT, Govt. of India for carrying out the research work.

## REFERENCES:

1. Frost HB, Soost RK. 1968. Seed Production:development of gametes and embryos. In: W Reuther, LD Batchelor and HJ Webber (editors). The Citrus Industry. Vol II. Division of Agricultural Science, University of California, USA. pp. 292–320.
2. Timmer LW, Garsney SM, Graham JH. 2000. Compendium of Citrus diseases.2nd Edn. APS Press, St Paul Minn.
3. Das A, Mandal B, Sarkar J, Chaudhuri S. 2004. RAPD profiling of some elite clones of Mandarin orange (*Citrus reticulata* Blanco) in the North Eastern Himalayan Region of India. Journal of Horticultural Science and Biotechnology 79:850–854.
4. Deng ZN, Gentile A, Nicolosi E, Domina F, Vardi A, Tribulato A 1995. Identification of invivo and invitro lemon mutants by RAPD markers. Journal of Horticultural Science. 70(1): 117-125.
5. Machado MA, Colleta FD, Targn MLPN, Pompeu Jr. J (1996) Genetic relationship of Mediterranean mandarin (Citrus deliciosa Tenore) using RAPD markers. Euphytica. 92: 321-326.
6. Lima DR, Vilarionnos AD, Soares Filho W dos S, Barbosa C de J. 2000. Identification of citrus zygotic seedlings by use of RAPD markers. Revista Brasileira de Fruiticultura. 22:181–185.
7. Ling D, Gmitter FG, Duncan LW, Xiao XY (1994) Inheritance of Citrus nematode resistance and its linkage with RAPD markers in Citrus. HortScience. 29:1235-1241.
8. Cheng FS and Roose ML (1995) Origin and inheritance of dwarfing by Citrus rootstock *Poncirus trifoliate 'Flying Dragon'Journal of American Society of Horticultural Sciences. 120:286-291.*
9. Deng Z, Huang S, Xiao YS, Gmitter FG (1997) Development and characterization of SCAR markers linked to *Citrus* trizteza virus resistance gene from *Poncirus trifoliata.Genome* 40: 697-704.
10. Fang DQ, Federici CT, Roose ML (1997) Development of molecular markers linked to a gene controlling fruit acidity in citrus. Genome 40:841-849.
11. Tisserat B. 1985. Embryogenesis, organogenesis and plant regeneration. In: RA Dixon (editor). Plant Cell Culture, a practical approach. IRL Press, Oxford, UK. pp. 79–104.
12. Das A, Mandal B, Paul AK, Chaudhuri S. 2003. Standardization of germination for increased production of nucellar plantlets of Mandarin orange (*Citrus reticulata* Blanco) under controlled condition. Journal of Interacademicia 7:343–346.
13. Das A, Mandal B, Sarkar J, Chaudhuri S. 2005. Genetic resource survey of Mandarin orange (*Citrus reticulata* Blanco) in the north-eastern Himalayan region of India. Plant Genetic Resources Newsletter 143:34–39.

14. Kahangi EM, Lawton MA, Kumar CACY. 2002. RAPD profiling of some banana varieties selected by small-scale farmers in Kenya. Journal of Horticultural Science and Biotechnology 77:393–398.
15. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18:6531–6535.
16. Parlevliet JE, Cameron JW. 1959. Evidence on the inheritance of nucellar embryony in citrus. Procedings of the American Society of Horticultural Science 74:252–260.
17. Khan IA, Roose ML. 1988. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of Trifoliate orange. Journal of the American Society of Horticulture Science 113:105–110.
18. Soost RK, Williams TE, Torres AM. 1980. Identification of nucellar and zygotic seedlings of citrus with leaf isozymes. Hortscience 15:728–729.
19. Soares Filho, W dos S, Lee LM, Cunha Sobrinho A P da (1995) Infuence of pollinators on polyembryony in citrus. Acta Horticulturae. n. 403, p. 256- 265.
20. Rodriguez M, Monter A, Espinosa A, Castaneda CG, Velazquez A (2004). Polyembryony and identification of Volkameriana lemon zygotic and nucellar seedlings using RAPD. Pesqui Agropecu Bras Brazilia. 39:551-559.
21. Ghosh SP, Singh RB. 1993. Citrus in South East Asia. FAO/RAPA Publication no. 1993/24. FAO, Bangkok, Thailand. 70 p.

**TABLES AND FIGURES:**

**Table1:** RAPD analysis of four candidate seedlings (nucellar & zygotic of *C. reticulata* & *C. aurantifolia* with 20 decamer primers with hybrid discriminating ability

| **Primer Name** | **Primer Sequence** | **Total number of bands generated** | ***Citrus reticulata* nucellar** | ***Citrus reticulata* zygotic** | ***Citrus aurantifolia* nucellar** | ***Citrus aurantifolia* zygotic** |
|---|---|---|---|---|---|---|
| OPB12 | CCTTGACGCA | 3 | 3 | 3 | 2 | 0 |
| OPH04 | GGAAGTCGCC | 8 | 8 | 0 | 8 | 0 |
| OPN12 | CACAGACACC | 7 | 6(4 ab) | 5(1 4 ab) | 4(123 ab) | 0 |
| OPN06 | GAGACGCACA | 3 | 3 | 3 | 2 | 0 |
| OPA01 | CAGGCCCTTC | 4 | 4 | 4 | 3 | 0 |
| OPA18 | AGGTGACCGT | 3 | 3 | 2 | 1 | 0 |
| OPAT03 | GACTGGGAGG | 5 | 5 | 5 | 0 | 0 |
| OPD08 | GTGTGCCCCA | 6 | 6 | 1(5p) | 0 | 0 |
| OPM13 | GGTGGTCAAG | 0 | 0 | 0 | 0 | 0 |
| OPB01 | GTTTCGCTCC | 6 | 6 | 6 | 6 | 0 |
| OPA13 | CAGCACCCAC | 1 | 1 | 0 | 0 | 0 |
| OPC04 | CCGCATCTAC | 4 | 4 | 4 | 4 | 0 |
| OPA07 | GAAACGGGTG | 4 | 4 | 2(14ab) | 0 | 0 |
| OPA08 | GTGACGTAGG | 5 | 5 | 0 | 0 | 0 |
| OPH15 | AATGGCGCAG | 8 | 8 | 8 | 4(12678ab) | 3(345p) |
| OPM06 | CTGGGCAACT | 10 | 10 | 2(45P) | 5(34578p) | 0 |
| OPAT04 | TTGCCTCGCC | 4 | 4 | 0 | 3(4ab) | 1(2p) |
| OPN07 | CAGCCCAGAG | 6 | 5(1ab) | 5(1ab) | 4(46 ab) | 0 |
| OPC01 | TTCGAGCCAG | 4 | 4 | 4 | 0 | 4 |
| OPD07 | TTGGCACGGG | 5 | 5 | 5 | 0 | 0 |
| **20** | | **96** | 36 polymorphic bands | | 46 polymorphic bands | |

(p= present, ab=absent, band number indicated in bracket shows presence or absence)

**Table 2:** RAPD amplicons generated by Universal primers related to polyembryony

| Primer Name | Number of identified bands | Tentative Base pair of identified bands |
|---|---|---|
| OPN12 | 3 | 600,700,800 |
| OPD08 | 1 | 310 |
| OPA07 | 1 | 615 |
| OPH15 | 3 | 170,380,550 |
| OPM06 | 4 | 300,440,750,850 |
| OPN07 | 2 | 300,710 |

(Base pair calculation done with 100-1000 bp ladder)

**Figure 1:** RAPD profile generated with 20 decamer primers with 4 candidate seedlings of *C. reticulata* & *C. aurantifolia* (nucellar & zygotic)