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# Detection of Zygotic Embryos of *Citrus reticulata* by Random Amplified Polymorphic DNA Technique

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**Abstract:** ***In the present study, Random amplified polymorphic DNA (RAPD) markers were used to study the difference between the zygotic and nucellar embryos of highly polyembryonic Citrus reticulata plant type collected from North Eastern Himalayan (NEH) region. Twenty five decamer oligonucleotide primers were used to amplify genomic DNA extracted from leaf samples. A total of 62 strong, unambiguous amplicons were generated out of which 47 were polymorphic. Hierarchical Cluster analysis based on Squared Euclidean distance using nearest neighbor method of binary data with SPSS software, ver. 16.0 differentiated all the seedlings into two different groups. The seedlings with 100% similarity were regarded as nucellar or true to the mother type. Other seedlings with slightest difference were regarded as zygotic. Four arbitrary primers OPA18, OPH11, OPB10, OPAA10 were able to discriminate the zygotic and nucellar seedlings. In an open pollinated population with very less morphological distinction, RAPD analysis can rapidly identify the zygotic embryos helps to obtain genetically uniform plant types. This method saves resources and helps in execution of proper management practice to a small uniform population to fix beneficial heterosis for the poor farmers of North Eastern Himalayan region.***

**Keywords:** Citrus reticulata, polyembryony, zygotic, nucellar, RAPD, propagation

## 1. Introduction

Polyembryony is a genetically determined trait found in some of the angiosperm families of plants. It is a quantifiable trait and its extent varies from plant to plant. In a cross-pollinated plant like citrus the zygotic population carries the genetic load and creates heterogeneous segregated populations but the nucellar one reflects the genetic architecture of the mother plant. This differential phenomenon leads to the exploitation of polyembryony in propagation of *Citrus*. The genus *Citrus* have been considered as a model group for the study of polyembryony. This phenomenon has serious importance in crop improvement as it can be utilized to fix heterosis of a hybrid by the production of true-to-the parental type progeny in the easiest way.

The North Eastern India is recognized as the natural home and origin of different cultivated species of *Citrus*, their progenitors and primitive germplasms. The region suffers from decline syndrome. For rejuvenation of the *Citrus* orchards supply of suitable planting material is essential. Citrus plants go through a long juvenile stage and require 6-7 years to fruiting. The assessment of fruit quality and performance requires time. The poor farmers usually compelled to wait for long period to understand the performance of the plants. They usually keep the uniform population generated from a single selected clone and after some years rogue excess plants. Till the screening of actual clone they unnecessarily spent huge resources on rearing of excess seedlings. To reduce their financial investment an early screening of the seedlings is necessary.

The morphological variation [1] could not be correlated with embryological variation in *Citrus*. If the parents are diverse the leaf characters can be correlated with embryological variation but if the pollen parent is closely related the variation could not be detected morphologically [2]. In North Eastern Hilly region of India especially in Darjeeling district of West Bengal the seed population is open pollinated and the seed source is not known.

For early identification of the zygotic seedlings a reliable marking system is required which is not possible with morphological identification alone. Different molecular techniques such as isozyme [3], RAPD [4], [5], [6], SSR [7], ISSR [8], are available. For establishing a low-cost, farmer friendly, early detection method especially keeping the financial condition of the poor farmers of India here we chose RAPD technique for zygotic detection from a open pollinated population of *Citrus reticulata* from Mirik region of Darjeeling district of West Bengal.

## 2. Materials and Methods

One (*Citrus reticulata)* plant from Mirik, Darjeeling was marked for high productivity, regular bearing and fruit quality and palatability of the fruits. The particular clone is unique for its late maturing habit. When most of the Citrus orchards of Darjeeling become empty this particular clone produces fruits. Mature fruits from this particular clone were collected and brought to the laboratory at Kalyani, West Bengal. 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 Tisserat [9] and noted. 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. All the germinating embryos under the integument were taken out. More than one seedling developing from a single point by the fission of the original zygotic or nucellar embryo was 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 all the seedlings were of seven month old with sufficient leaves for DNA extraction was available, 15 seedlings with good growth were selected as elite clones of the mother for further molecular analysis. Leaves of mother plant is brought to laboratory in controlled condition (-20 degree C) in Cool box from Mirik and kept at -20 degree Freezer for further DNA extraction and comparison of the seedlings with the mother.

## 3. DNA Extraction And RAPD Analysis

Variability in banding pattern among the seedlings developed from a single fruit was investigated with Random Amplified Polymorphic DNA (RAPD) analysis. The mother plant DNA was also used for RAPD analysis. The experiment was carried with 25 Operon decamer primers selected by preliminary screening to give polymorphism and reproducible fragment patterns in both the species [10]. Genomic DNA was extracted from the soft leaves of the seedlings using CTAB protocol [11]. The quantity and amount of DNA were determined as described by Kahangi et al. [12]. Amplification was achieved by the protocol outlined by Williams et al. [13], with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X PCR´ 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 (Bangalore Genei), which was run along with the amplified products. The primers that could generate differential banding patterns of the seedlings of different embryonic origins of a single fruit were noted along with the mother plant. Squared Euclidean Distance was calculated using a binary matrix of RAPD data and Dendogram was constructed using Nearest Neighbour Method with SPSS software, ver. 16.0.

## 4. Results and Discussion

North Eastern Hilly region of India is one of the centres of origin of *Citrus reticulata*. *Citrus* plants are mainly propagated through seed in this region from old days. Each seed give rise to several embryos and those embryos are used as propagating material. The farmers practise rouging off type seedlings. Representative embryos from the open pollinated seed population were evaluated to find suitable marker for identifying the zygotic seedlings.

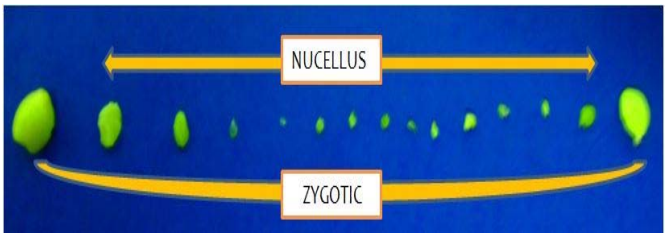


**Plate1:** Showing presence of zygotic and nucellar embryos in seeds of *Citrus reticulata* collected from lower Mirik

Seeds 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 data was recorded. Here from each seed the embryos were taken out and numbered with respect to each seed. No inference was drawn regarding the nature of the embryo at early stage though a recording of zygotic and nucellar embryos were done for comparing the morphological identification with molecular marking. Twins were recorded in this population.

Twin and triplet zygotic embryos were reported in *Citrus reticulata* [14]. Morphological separation of the seedlings originating from zygotic and nucellar embryos of *Citrus reticulata* usually shows zygotic: nucellar ratio of 1:3. The seedlings were grown in separate pots with addition of organic matter and coir pith. The DNA extracted from those 15 healthy seedlings from a single fruit and their mother when run with 25 selected RAPD primers gave some interesting results.

After preliminary screening 19 primers yielding strong, intense, unambiguous and reproducible DNA fragments were selected. The list of the selected primers, their sequences, maximum number of fragments obtained and range of the size of the fragments were as shown in Table 1. The amplified fragments varied from 1 to 9 (Table1). The size of the fragments ranged from 100 bp to 750 bp and a total of 62 amplicons were generated. The citrus genome was about 563 mbp [15]. The analysis based on 62 markers was expected to saturate the genome at a density of one marker for every 9.08 mbp, which appeared to be adequate to make meaningful statements about the diversity or relatedness among the seedlings of mandarin orange plant

types with respect to the trait polyembryony [16]. Each set of PCR reaction accompanied positive and negative control. In every PCR reaction no DNA fragments were found in the negative control while similar banding patterns were found in the positive control indicating contamination-free PCR ingredients and the consistency of the protocol. OPAA10, OPZ10, P140, OPA04 were able to generate more bands with high level of polymorphism. As an attempt was taken to differentiate zygotic and nucellar embryos by appropriate primers here more emphasis was given to known primers those were able to generate polymorphism. The PIC value of the primers reflects OPA04 and OPAA10 are best for discrimination seedlings of *Citrus reticulata*.

Rao et. al.[17] reported five primers out of which OPAA10 gave optimum result in this experiment. Ochoa et. al. [18] reported ten primers OPA-01, 02, 04, 11, 18, OPB-06, 07, 10, 12 and SAP-04 able to identify polyembryony in *Mangifera*. SAP01 and SAP04 were specially constructed by the researcher to discriminate embryos of *Mangifera* as *Citrus* and *Mangifera* are important horticultural fruit crops showing polyembryony. OPA-02, OPA-11, OPA-18 gave polymorphism in both the fruit crops. Both SAP 1 and 4 were constructed by Ochoa (2012) team especially for *Mangifera* embryo discrimination. Those two primers were included in this experiment. SAP-01 was not able to amplify any product. SAP-04 primer amplified only monomorphic amplicon in *Citrus* whereas this primer gave maximum number of bands in *Mangifera*. The amplicon was present in all the fifteen seedlings of *Citrus*. This finding reveals that the polyembryony locus of *Citrus* and *Mangifera* may have some difference. Primer OPV10 and P140 were able to characterize seedling 1 with 590 and 495 base pair amplicons.

**Table 1:** Numbers and Fragment Sizes of Amplicons Obtained from 15 *Citrus reticulata* Seedlings Of NEH Using 15 Decamer Random Primers.

| *Primer Name* | *Total number of band* | *Polymorphic Amplicons* | *polymorphism %* | *Range of Amplicon (Base pair)* | *Common Amplicon (Base pair)* | *Polymorphic Information Content (PIC)* |
|---|---|---|---|---|---|---|
| OPA18 | 3 | 2 | 66.66667 | 385-590 | 385 | 0.2467 |
| SAP04 | 1 | 0 | 0 | 300 | 300 | 0 |
| OPH11 | 1 | 1 | 100 | 580 | 580 | 0.32 |
| OPAD10 | 5 | 4 | 80 | 340-680 | 450 | 0.324 |
| OPM10 | 4 | 3 | 75 | 390-680 | 390 | 0.35 |
| OPV10 | 4 | 3 | 75 | 320-530 | 320 | 0.245 |
| OPZ10 | 2 | 2 | 100 | 290-400 | 290 | 0.18 |
| P140 | 4 | 4 | 100 | 100-490 | 100 | 0.325 |
| OPAA10 | 9 | 9 | 100 | 200-690 | 410 | 0.4025 |
| OPA04 | 5 | 5 | 100 | 190-600 | 190 | 0.448 |
| OPA11 | 4 | 4 | 100 | 300-530 | 400 | 0.348 |
| OPAA02 | 3 | 2 | 66.66667 | 160-370 | 160,370 | 0.12 |
| OPB07 | 3 | 3 | 100 | 170-470 | 170 | 0.46 |
| OPB08 | 2 | 2 | 100 | 100-290 | 290 | 0.18 |
| OPAT04 | 4 | 4 | 100 | 320-750 | 700 | 0.335 |
| OPB17 | 3 | 3 | 100 | 360-720 | 720 | 0.28 |
| OPB10 | 3 | 3 | 100 | 220-500 | 500 | 0.18 |
| OPM05 | 1 | 1 | 100 | 300 | 300 | 0.18 |
| OPB02 | 1 | 1 | 100 | 350 | 350 | 0.18 |

OPA11 with a 300 base pair fragment is able to detect uniqueness of seedling 2. Seedling 4 is specified by 280 base pair amplicon generated by OPAA02 primer. OPAA10 and OPB10 were useful for identification of nucellar embryos with cent percent similarity.

## 5. Identification of zygotic and nucellar seedlings using RAPD

Polymorphism was clearly observed among fifteen seedlings with selected decamer primers. A total of 62 bands were generated, and 72.8% were polymorphic. A Genetic similarity Matrix was calculated from the presence and absence of 62 RAPD bands obtained from 15 seedlings by 15 primers according to Squared Euclidean Distance as stated in Material and method part. The matrix estimated all pair wise differences in the amplification products. Based on this matrix, the highest (100%) genetic similarity was observed among the plants S7, S10, S11, S12, S13, S14 and S15.

**Table 2:** Primers Efficient To Detect Individual Hybrids and Nucellar Seedlings

| Seedling | Unique Band | | | |
|---|---|---|---|---|
| S1 | OPV10 590 bp, P140 495 bp | | | |
| S2 | OPA11 300 bp | | | |
| S4 | OPAA02 280 bp | | | |
| S7, S9,S10,S11,S12,S13, S14, S15 | OPAA10 250bp | OPA18 470bp | OPB10 220bp | OPH11 480 bp |

These seven seedlings are regarded as nucellar due to their similarity. Eight seedlings were different from the rest seven. The cluster analysis of the RAPD data using minimum variance algorithm and nearest neighbour method created the dendrogram as shown in Figure 1.

**Figure 1:** Dendogram based on genetic distance, summarizing the data on differentiation between the zygotic and nucellar seedlings according to RAPD analysis

OPAA10, OPA18, OPH11 and OPB10 were able to differentiate the zygotic seedlings from the nucellar ones representing the genetic architecture of the mother plant. OPA18 was also able to characterize three hybrids with similar amplicons and four with a slight difference. OPA 18 proved very useful both for discriminating nucellar from zygotic and also for distinguishing hybrids from one another. OPH 11 primer with very low PIC was not excluded due to high discriminating ability. OPB10 helped in grouping of nucellar in a single cluster with generation of a single band different from the rest. OPM10 and OPV10 were useful for discrimination of zygotic seedlings.

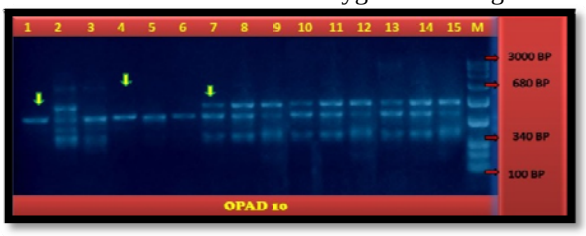


Plate 2a

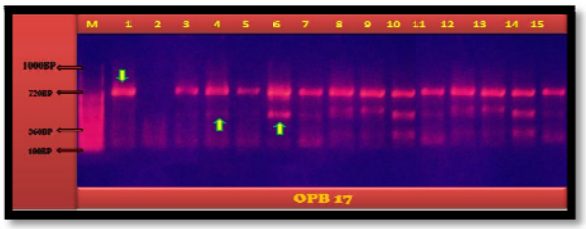


**Plate 2a & b:** Showing RAPD profile of a total of 15 seedlings including zygotic & nucellar types of Citrus reticulata along with a 100 bp ladder

Arbitrary primer OPAA10 with high PIC value is a good selection for differentiate nucellar. The band pattern in zygotic plants was different from that of the mother plant, due mostly to the absence of some fragments, as well as to the presence of markers (Table 2) in zygotic seedlings.

**Table 3:** RAPD Primers Able To Differentiate Zygotic & Nucellar Seedlings

| *Primer Name* | *Zygotic Seedlings* | *Nucellar Seedlings* |
|---|---|---|
| OPA18,OPH11, OPB10,OPAA10 | 1,2.3,4,5,6,8,9 | 7,10,11,12,13,14,15 |

No single primer was able to identify all zygotic seedlings. This coincides with results obtained by Vilarinhos et al. [19], who identified 12 'Volkameriano' lemon x 'Cravo' lemon hybrid plants, previously labelled as zygotic due to morphological characteristics; out of the 20 primers tested only six produced banding patterns that discriminated between the parents.

One important observation is the zygotic and nucellar seedling survival. The molecular analysis revealed 53% of the seedlings are zygotic in origin and 47% are nucellar which is different from the value of 18% and 80% obtained by morphological screening. In *C. volkameriana*, 88% of seedlings from monoembryonic seeds and 26% of seedlings from polyembryonic seeds were classified as zygotic by the RAPD technique. Several factors like environment, food supply, pollination, pollen source and genetic regulation etc. were reported to influence polyembryony in citrus. Nasharty [20] reported that geographical location had influence on nucellar embryo formation in citrus. He observed more number of embryos in citrus seeds when grown at Los Angles than at Riverside in the same season. Temperature was considered as a factor influencing the production of extra embryos by earlier experiments. The reduction in the number of embryos per seed was reported due to cessation of division in nucellar cells in the glass house due to higher temperature than at the field by Nakatami *et al.* [21]. They also suggested that cross-pollination of polyembryonic seed parents under high temperature might be beneficial for citrus breeding, to overcome the hurdle of polyembryony. Type of pollination might also have some role, particularly in the degree of polyembryony. Cross-pollination was recognized to give higher percentage of polyembryony than self-pollination (Cekvava, 1968), cited in [22]. However, Wakana and Uemoto [23] proposed that nucellar embryos could be generated without pollination but failed to develop due to lack of endosperm development. Orientation of branches in the tree was also suspected to play some role to affect nucellar embryo formation. Furusato and Suzuki [24] reported that seeds from the northern side of the tree had higher mean number of embryos than those from southern side. Nucellar embryony was found as an inheritable genetic character, controlled by one or more number of genes [25].

These genes might have the role to regulate the potent inhibition of embryogenesis in nucellus cells of mono-embryonic citrus varieties. It was also stated that seed storage protein was involved in this phenomenon [26]. Zheng and Cheng [27] proposed that the gene expression and regulation of zygotic embryo and nucellar embryo development were the key to resolve the mechanisms of embryogenesis. Koltunow *et al.* (1995) reported that during fruits development, the timing and sequence of the early events of nucellar embryo formation were synchronous in seeds and unfertilized ovules which indicated a coordinated control of embryo development in spatially and developmentally distinct structure. Cristofani & Machado [28] reported 6% zygotic seedlings of 'Cravo' lemon in a sample of 50 plants taken from a population of 576 produced in a greenhouse. The difference in these results is due to the fact that embryos of each seed were separated and cultivated in vitro, using a baby food jar for each seed, which allowed us to obtain a seedling from every embryo. Different environment, i.e., greenhouses [28]; [29], germination containers [30], pots [31], or germinating the seed in vitro [29] gave different germination rate. When germination is carried out in a substrate, not all embryos within a seed develop into seedlings, since many of them are dehydrated or do not have enough reserve material in order to seedling growth and development.

In this research work an attempt was taken to differentiate nucellar and zygotic seedlings at molecular level. Previous literature stated the morphological separation of zygotic and nucellar embryo is not always correct. The detection of zygotic twin and triplet in mandarin orange population actually complicates the process. The poor farmers of North Eastern Hilly region usually scatter seed in seed bed and rogue the off type seedling. The occurrence of significant number of zygote abnormality could decrease the nucellar percentage and may complicate its identification. In an open pollinated population the morphological identification of nucellar embryos become more difficult. Keeping these points in mind DNA based molecular marking system is used for differentiation of hybrids from the nucellar. Four RAPD primers were efficient to distinguish the seedlings into two distinct clusters. One cluster comprises all the nucellar seedlings and others (zygotic seedlings) joining the cluster sequentially. Some more primers were also found with capacity to express the difference among zygotic embryos. RAPD is the cheapest DNA marking system and within tenure of 3 to 4 days the decamer primers can identify the nucellar seedlings and aid in establishment of uniform population. The selected primers could be used for uniform *Citrus reticulata* plant type selection especially for North Eastern Himalayan region of this sub continent and also for other region.

## 6. Future Scope of Research

The information generated in this paper is the foundation for molecular marking of polyembryony character of *Citrus reticulata* in West Bengal. The work could be elaborated with a large population size or with a true hybrid developed by hybridization to truly help the poor farmer's of Darjeeling region. Citrus is a model crop for polyembryony and this work is an initiation of molecular marking of this specific trait. This work may be elaborated for zygotic and nucellar differentiation in other Citrus species along with some other crops with plural embryo. The amplicons generated in this work could be sequenced to construct more precise marker. An elaborate marker validation trial may help in development of uniform population for the farmers of North Eastern region of India.

## 7. Acknowledgement

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## Author Profile

**Dr. Bidisha Mondal** was graduated from University of Calcutta with Botany Honours and was a gold medallist in Genetics & Plant Breeding (Ag.) from University of Calcutta. She was selected for training in Plant Biotechnology: Molecular Marker Technology by DST, Govt. of India among the 10 researcher from all over India. She has worked as guest research worker in Bose Institute after completing her Master's degree. She obtained doctorate degree from Bidhan Chandra Krishi Viswavidyalaya in 2005 with funding by a ICAR-NATP-CGP project of Govt. of India. She has 17 publication in indexed journal out of which 4 are internationally highly reputed one. She has 6 full papers in national &international level proceedings. Adding to this she has written 5 chapters of Subsidiary Botany Book of Netaji Subhas Open University and edited two books of Genetics and Evolution of the same University. Other than that she has written 5 popular articles in Bengali magazines. She has a research career of more than 13 years and a teaching career of 5 years. She served Netaji Subhas Open University as Assistant Professor in Botany from 2009 to 2012 and also taken classes of Post Graduate Diploma in Medicinal & Aromatic Plants of the same University. As Guest Lecturer she took classes of Molecular Biology, Biotechnology & Microbiology. At present she is working as Scientist in Bidhan Chandra Krishi Viswavidyalaya. She has earned the prestigious Bio-CARe Women Scientist award of Department of Biotechnology, Govt. of India in 2012. She has delivered several radio lectures as anchor and distance teacher on several socio-scientific issues. She is the life member of Indian Science Congress Association. Her research interest involves biotechnology, molecular biology, genetics, plant physiology, plant nutrition and environment.

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