

Figure 1: Dendrogram 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.

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 Angeles 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].

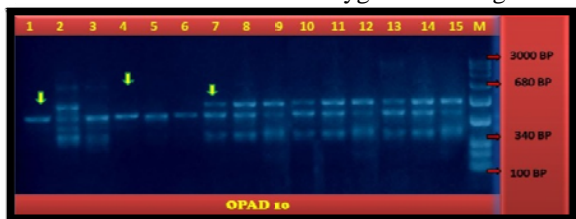


Plate 2a

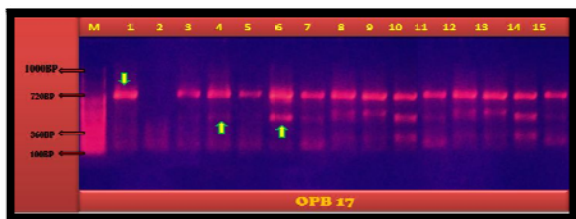


Plate 2a & b: Showing RAPD profile of a total of 15 seedlings including zygotic & nucellar types of Citrus reticulata along with a 100 bp ladder

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

These genes might have the role to regulate the potent inhibition of embryogenesis in nucellar 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 'Crayo' 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.

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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.



Mr. Apurba Pal has graduated from Biswa Bharati University with Agriculture and obtained Masters from Bidhan Chandra Krishi Viswavidyalaya securing first position in Plant Physiology. He is interested in Photobiology, hydroponics, plant nutrition. He has learned molecular techniques during his masters.



Dr. Ramkrishna Saha have awarded Ph. D degree in Plant Pathology from Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, W.B. in the year 2004 preceded by M.Sc.(Ag) Hons. in Plant Pathology in 1998 and B.Sc.(Ag) Hons. in 1996 from the same University. He also qualified ICAR NET in 2002 and presently working as Assistant Director of Agriculture at Department of Agriculture, Govt. of W.B. He is engaged in his current employment since 12 years where doing the Agriculture Extension work and implementations of different Agriculture related schemes of Govt. of India and State Govt. The author at the same time continued his laboratory research. He possesses six numbers of other research publications one book chapters in peer reviewed journals. The author is the life member of Indian Science Congress. The area of research interest is in the field of biotechnology and nutritional management in disease development.