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RESEARCH ARTICLE

Microsatellite Markers based Identification of Zygotic and Nucellar Embryos in Polyembryonic Mango (*Mangifera indica* L.) Genotypes Vellaikulamban and Olour

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Abstract

The nucellar seedlings from polyembryonic mango seeds are useful as clonal rootstock while zygotic seedlings could be valuable for genetic variability. However, discriminating zygotic from nucellar seedlings originating from a single kernel based on morphology is impractical. The current study aimed to differentiate zygotic and nucellar embryos among multiple embryos in polyembryonic genotypes Vellaikulamban and Olour using nine SSR markers. Observed heterozygosity ranged from 0.182 (MiIIHR23) to 1.0 (MiIIHR17). LMMA12 had the maximum (0.823) polymorphic information content (PIC), and MiIIHR23 the minimum (0.562). Dendrogram using the neighbor-joining method demonstrated that embryos developed from nucellus grouped in the same cluster with the maternal parent, whereas zygotic embryos clustered in a distinctly different group. Six SSR markers identified the farthest embryo from the funicular point as zygotic in Olour, while in Vellaikulamban some discrepancy was observed for two markers.

Keywords: Polyembryony, Mango, Nucellar, Zygotic, Embryos, SSR, Vellaikulamban, Olour.

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Introduction

Mango is a highly heterozygous and heterogeneous fruit crop that is propagated mainly by seed and grafting. Based on the kernel morphology, mango varieties are generally classified into two groups, namely monoembryonic and polyembryonic types. Monoembryonic mango varieties yield one seedling per seed which is expectedly zygotic in origin. Polyembryonic varieties often yield many seedlings. When multiple seedlings are produced, one of them is considered zygotic in origin, and the rest is from the maternal tissue (Viruel *et al*., 2005). Generally, the nucellar seedlings are used as rootstock for crop production whereas the weaker single zygotic seedling is discarded during propagation. The major challenges were faced during the selection of nucellar seedlings from zygotic ones by researchers and nurserymen. A plantlet origin was distinguished morphologically based on the seedling's vigorousness by many workers in the polyembryonic mango (Sachar and Chopra, 1957; Cordeiro *et al.,* 2006). The formation of all vigorous seedlings due to dominant nucellar growth over a weaker zygotic seedling was reported by Srivastava *et al.* (1988) in mango. Recently, some contradictory results related to the origin of multiple seedlings in the polyembryonic mango seeds were reported (Ochoa *et al*., 2012; Rocha *et al*., 2014) when molecular markers were employed. These include zygotic seedlings

originating first and being more vigorous in growth over following nucellar seedlings in the polyembryonic mango variety 'Uba' using ISSR markers by Rocha *et al*. (2014) and occurrence of varying percentages of zygotic embryos in the polyembryonic mango varieties by Ochoa *et al.* (2012) and Rocha *et al*. (2014). Differentiation of nucellar and zygotic seedlings through molecular markers was attempted *viz*., RAPD (Cordeiro *et al*., 2006; Srivastava *et al*., 2010; Ochoa *et al*., 2012), ISSR (Srivastava *et al*., 2010; Rocha *et al*., 2014), DAMD (Srivastava *et al*., 2010), SSR (Ruiz *et al*., 2000; Viruel *et al*., 2005; Sane *et al*., 2011; Kumar *et al*., 2020; Judith *et al*., 2021) but most of the results of these findings were not in agreement to each other in mango itself. With this background, the current study was planned to identify a polyembryonic kernel's zygotic and nucellar embryos using SSR markers.

Materials and Methods

DNA Isolation from Selected Genotypes

The open-pollinated ripened fruits were harvested from the trees of selected polyembryonic mango genotypes, namely Vellaikulamban and Olour during 2020-21. Thereafter, the seeds were extracted from fruits, and the hardy endocarp and tegmen from the kernel surface were removed. The kernels were washed using 70% alcohol and later washed with distilled water. The multiple segments (kernel embryos) of each polyembryonic kernel were separated individually and subsequently, each separated individual embryo of the kernel was considered as a sample, labeled sequentially to their position in the funiculus (1st, 2nd, 3rd, and 4th embryos were numbered from the funicular point in counter clockwise and then coded as A, B, C, and D) (Table 1) for DNA isolation. Usually, the polyembryonic mango kernel is compacted with more embryos (up to 10 in Olour). In the current study, polyembryonic kernels comprising up to 3 to 5 embryos were selected for ease of separating individual embryos and enabling the isolation of DNA from each embryo.

The matured leaves sample of selected mango mother trees of Vellaikulamban and Olour and their respective kernel embryo samples were collected for genomic DNA extraction using a CTAB (cetyltrimethylammonium bromide) based method described in Ravishankar *et al*., 2000. The quantity of extracted DNA was checked using a spectrophotometer (Gene Quanta, Amersham Biosciences, Holliston, USA) and the quality was checked through electrophoresis in 0.8% agarose gel.

PCR Conditions and Analysis

SSR markers were amplified using a fluorescence-based M13-tailed PCR reaction (Oetting, 1995) and the typical M13 tail was added to the SSR primers at the 5th position of the primer sequence (Schuelke, 2000). A total of nine SSR primers (Ravishankar *et al*., 2011) (Table 2) were used to genotype 22 mango samples (Table 1). The PCR was done in a 20 µL reaction volume comprising 2 µL of 20 ng DNA, Taq buffer 10X (2 µL, tris pH with 15 mM $MgCl_2$), dNTPs (1-µL of 10 mM), a forward primer of m-13 tail (1-µL of 5 pM), reverse primer (1-µL of 5 pM), 5 pM concentrated 1-µL fluorescent probes FAM, VIC, NED, and PET M13 primers, Taq polymerase (0.3 µL) and nuclease-free water (11.2 µL). A Bioer Life Pro Thermal Cycler was used to execute all of the PCR experiments (Bioer Technology, China). The PCR amplification condition was as follows: initial denaturation at 94°C for 1-minute, followed by 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 30 seconds), and extension (72°C for 1-minute) steps, and a final extension at 72°C for 5 minutes.

On an automatic 96-capillary automated DNA sequencer (ABI 3730 DNA Analyzer, Applied Biosystems, USA), the amplified products labeled with FAM, VIC, PET, or NED were multiplexed and pooled before separation. To identify the exact fragment size of the PCR product, the data was further analyzed using the Peak Scanner software (Applied Biosystems, USA). The fragment size data were utilized to calculate the number of alleles per locus, observed

Table 1: Mango samples used in the study

Sample. No.	*Samples*	*Sample No.*	*Samples*
1	Vellaikolumban mother plant - VK-M	12	Olour mother plant - OL-M
2	Kernel-1 1st embryo (VK-1A)	13	Kernel -1 1st embryo (OL-1A)
3	Kernel -1 2nd embryo (VK-1B)	14	Kernel -1 2nd embryo (OL-1B)
4	Kernel -1 3rd embryo (VK-1C)	15	Kernel -1 3rd embryo (OL-1C)
5	Kernel -2 1st embryo (VK-2A)	16	Kernel -1 4th embryo (OL-1D)
6	Kernel -2 2nd embryo (VK-2B)	17	Kernel -1 5th embryo (OL-1E)
7	Kernel -2 3rd embryo (VK-2C)	18	Kernel -2 1st embryo (OL-2A)
8	Kernel -3 1st embryo (VK-3A)	19	Kernel -2 2nd embryo (OL-2B)
9	Kernel -3 2nd embryo (VK-3B)	20	Kernel -2 3rd embryo (OL-2C)
10	Kernel -3 3rd embryo (VK-3C)	21	Kernel -2 4th embryo (OL-2D)
11	Kernel -3 4th embryo (VK-3D)	22	Kernel -2 5th embryo (OL-2E)

Table 2: SSR primers used in the study.

Primers ID	*Primers (5'–3') sequence*
LMMA9	F- TTGCAACTGATAACAAATATAG
	R- TTCACATGACAGATATACACTT
LMMA10	F- TTCTTTAGACTAAGAGCACATT
	R- AGTTACAGATCTTCTCCAATT
LMMA12	F- AAAGATAGCATTTAATTAAGGA
	R- GTAAGTATCGCTGTTTGTTATT
MillHR29	F- CAACTTGGCAACATAGAC
	R- ATACAGGAATCCAGCTTC
MillHR17	F- GCTTGCTTCCAACTGAGACC
	R- GCAAAATGCTCGGAGAAGAC
MillHR18	F- TCTGACGTCACCTCCTTTCA
	R- ATACTCGTGCCTCGTCCTGT
MillHR23	F- TCTGACCCAACAAAGAACCA
	R- TCCTCCTCGTCCTCATCATC
MillHR-24	F- GCTCAACGAACCCAACTGAT
	R- TCCAGCATTCAATGAAGAAGTT
MillHR-26	F- GCGAAAGAGGAGAGTGCAAG
	R- TCTATAAGTGCCCCCTCACG

heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) value using Cervus 3.0 software, (Kalinowski *et al*., 2007). The results from the peak scanner were utilized to create barcodes. The barcodes were developed using a total of nine SSR markers and developed by Ravishankar *et al*. (2011) and Ravishankar *et al*. (2015) and Ravishankar *et al*. (2015). Short genetic sequences from a typical portion of the genome are used in barcoding. The 'Barcode of Life Database (BOLD, maintained by the University of Guelph) was used for all 20 mango samples. The neighbour-joining approach (NJ) was used to create a dendrogram for 22 mango samples using Darwin software (Perrier *et al*., 2003; http://darwin.cirad.fr/darwin).

Result and Discussion

Genetic Analysis of SSR Primers

The molecular analysis revealed that the selected SSR primers amplified alleles across all the samples and showed diverse levels of polymorphism. Among nine SSR markers, LMMA12 had a high amount of polymorphism (PIC = 0.823), while MilIHR23 had a low level of polymorphism (PIC = 0.562). The allele sizes and summary statistics of the SSR

Table 3 (a): Gene scan analysis of the Vellaikulamban mother plant and its progeny kernel embryos yielded PCR product sizes (bp) for nine SSR primers.

Primers ID	*Mother tree*	*Kernel-1*			*Kernel-2*			*Kernel-3*			
	VK-M	*VK-1A*	*VK-1B*	*VK-1C*	*VK-2A*	*VK-2B*	*VK-2C*	*VK-3A*	*VK-3B*	*VK-3C*	*VK-3D*
LMMA9	105/107	106/107	106/106	104/106	104/104	104/106	105/105	106/106	105/105	108/108	106/107
LMMA10	102/104	102/103	102/105	104/105	103/104	103/104	102/105	104/105	105/105	102/105	102/105
LMMA12	104/105	102/105	102/105	105/106	104/105	103/105	103/105	105/106	104/105	103/105	105/105
MillHR29	109/109	109/109	109/109	108/109	108/109	109/109	109/109	109/109	109/110	109/109	109/109
MillHR17	108/109	108/109	108/109	108/109	108/109	108/109	108/110	109/110	109/110	108/109	109/110
MillHR18	108/109	109/109	109/111	109/109	108/108	107/109	109/109	107/109	108/108	102/104	108/110
MillHR23	105/106	106/106	105/105	105/105	104/105	105/105	105/105	104/104	104/104	105/105	105/105
MillHR24	105/107	105/107	108/108	108/108	105/106	105/108	105/108	108/108	108/110	108/110	108/108
MillHR26	109/111	109/09	110/111	109/110	110/110	106/107	105/105	110/110	109/110	108/110	104/105

Table 3 (b): Gene scan analysis of the Olour mother plant and its progeny kernel embryos yielded PCR product sizes (bp) for nine SSR primers.

Primers ID	*Mother tree*	*Kernel-1*					*Kernel-2*				
	OL-M	*OL-1A*	*OL-1B*	*OL-1C*	*OL-1D*	*OL-1E*	*OL-2A*	*OL-2B*	*OL-2C*	*OL-2D*	*OL-2E*
LMMA9	104/106	106/106	106/106	104/105	104/104	114/115	105/105	106/106	105/106	105/105	105/107
LMMA10	108/109	107/108	108/108	108/108	107/109	117/119	107/107	108/110	108/108	108/108	108/108
LMMA12	109/111	109/110	109/110	109/112	109/109	115/117	109/110	110/112	110/110	110/111	109/110
MillHR29	104/106	104/104	105/105	105/106	106/106	120/120	105/106	105/105	106/107	105/105	104/106
MillHR17	108/110	108/109	109/110	108/109	108/109	108/110	108/109	108/109	109/110	107/109	109/110
MillHR18	108/109	108/108	107/109	110/110	107/109	107/109	107/107	110/110	107/107	106/107	106/108
MillHR23	106/106	105/105	105/105	104/106	105/105	110/112	105/105	104/104	104/104	106/106	105/105
MillHR24	105/107	106/108	105/107	107/107	106/108	108/110	105/107	108/108	108/108	105/108	106/106
MillHR26	109/110	109/109	110/110	109/109	109/109	113/113	109/112	109/110	111/111	108/109	109/110

Table 4: List of SSR primers and their characteristics based on genetic analysis of polyembryonic kernel embryos of Vellaikolumban and Olour along with the mother plants.

Primer ID	*Number of alleles per locus (k)*	*Observed heterozygosity (Ho)*	*Expected heterozygosity (He)*	*Polymorphic information content (PIC)*
LMMA9	7	0.455	0.766	0.710
LMMA10	10	0.682	0.851	0.813
LMMA12	11	0.864	0.86	0.823
MilIHR29	8	0.364	0.759	0.712
MilIHR17	4	1	0.67	0.586
MilIHR18	8	0.545	0.797	0.746
MilIHR23	5	0.182	0.631	0.562
MilIHR24	5	0.636	0.725	0.669
MilIHR26	10	0.5	0.807	0.761
Mean	7.5	0.581	0.762	0.709
Range	4 to 11	0.182 to 1.00	0.631 to 0.851	0.562 to 0.823

markers are given in Tables 3(a), (b), and 4. At nine SSR loci, at least an allele of the maternal parent was found in most of the seed embryos except for a few embryos that lack a maternal allele. This validates the genetic composition of embryos that were similar to the mother parent and those that were not, which was the major objective of this study. Microsatellite markers are the most reliable and robust because of their co-dominant, multiallelic nature and as well as amenable to multiplexing (Powel *et al*., 1996; Collard *et al*., 2005). The gene scan analysis of 22 samples using 9 SSR markers detected an average of 7.5 alleles per locus, ranging from 4 alleles in MilIHR17 to 11 alleles in LMMA12. Ravishankar *et al*. (2015) found 23.37 mean alleles per locus (K), while Ravishankar *et al*. (2017) found 16.6 alleles per locus and concluded that a genotype's heterozygosity determines the number of alleles per locus. Recently, similar work done by Sajana *et al*. (2021) used 16 SSR markers for 6 polyembryonic mango samples and achieved similar results of 9.25 alleles per locus. The expected heterozygosity ranged from 0.631 in MilIHR23 to 0.851 in LMMA10. The observed heterozygosity ranged from 0.182 in MilIHR23 to 1.00 in MilIHR-17 indicating high polymorphism. The PIC value was maximum (0.823) for LMMA12 and minimum (0.562) for the MilIHR23 marker. All of the primers used in this study were found to be highly informative, reproducible, and polymorphic in distinguishing the maternal and zygotic embryos (Eiadthong *et al*., 1999).

Prediction of Embryo Origin based on Allelic Similarity

Nine SSR markers were used to identify the origin of the embryos based on the allelic size of parental plants of polyembryonic genotypes with their progeny kernel embryos. With the comparison of the maternal parent in both polyembryonic cultivars Vellaikulamban and Olour, most of the embryos were observed as a nucellar origin, while a few polyembryony seeds produced one zygotic embryo per kernel (VK-2B, VK-2C, VK-3C, VK-3D, and OL-1E) with different SSR profiles [Table 3 (a, b) and 5]. The current results of the molecular study confirm the findings of Kumar *et al*. (2020) who reported the frequency of one zygotic seedling per polyembryonic mango seed. When compared to the Vellaikulamban mother plant, VK-2B and VK-2C embryos were identified as zygotic embryos by the MilIHR26 marker, while VK-3C (3rdembryo of kernel no. 3) was indicated as the zygotic by MilIHR18 marker and another embryo VK-3D was also identified as a zygotic embryo through MilIHR26 marker; however, the rest of the Vellaikulamban embryos were identified as nucellar origin based on the SSR profile (Table 3a and 5). When compared to the Olour mother plant, the embryo OL-1E (5th embryo of kernel no.1) was identified as the zygotic origin with the six markers LMMA9, LMMA10, LMMA12, MilIHR29, MilIHR23, and MilIHR26 (Table 3b and 5). All markers confirmed that all the embryos of Olour second kernel (OL-2A, OL-2B, OL-2C, OL-2D, and OL-2E) were nucellar (Table 5).

Distance Matrices

The dendrogram created using the neighbour joining approach revealed three major clusters among 22 samples (Figure 1). Cluster I contain two subclusters in which, the embryos such as OL-1A, OL-1D, OL-2A, and OL-2E were found closely related and genetically similar to OL-M. The second subcluster of cluster I includes two sub-subclusters in which embryos *viz* OL-2B, OL-1C, OL-2C, and OL-2D were found closely related but distantly related to the OL-1B embryo. Cluster II contains two subclusters: the Vellaikulamban mother plant and their genetically identical nucellar embryos. The first subcluster of cluster II contains embryos viz VK-3D, VK-1B, VK-3C, VK-2C, VK-3B, VK-3A, VK-2B, VK-1C, VK-2A. The second subcluster included the VK mother plant along with the embryo VK-1A. Overall, cluster II revealed that most markers (excluding MilIHR26 and MilIHR18) could identify nucellar embryos similar to the Vellaikulamban

Table 5: SSR primers differentiated the zygotic and nucellar origin of polyembryonic kernel embryos of Vellaikulamban and Olour [Table 5. constructed using gene scan analysis data from Table 3(a) and 3(b)]

Primers ID	M-tree	Kernel-1			Kernel-2			Kernel-3				M-tree	Kernel-1				Kernel-2					
	VK-M	VK-1A	VK-1B	VK-1C	VK-2A	VK-2B	VK-2C	VK-3A	VK-3B	VK-3C	VK-3D	OL-M	OL-1A	OL-1B	OL-1C	OL-1D	OL-1E	OL-2A	OL-2B	OL-2C	OL-2D	OL-2E
LMMA9	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N
LMMA10	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N
LMMA12	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N
MilIHR29	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N
MilIHR17	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MilIHR18	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N	N	N	N	N	N	N	N
MilIHR23	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N
MilIHR24	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
MilIHR26	N	N	N	N	N	Z	Z	N	N	N	Z	N	N	N	N	N	Z	N	N	N	N	N

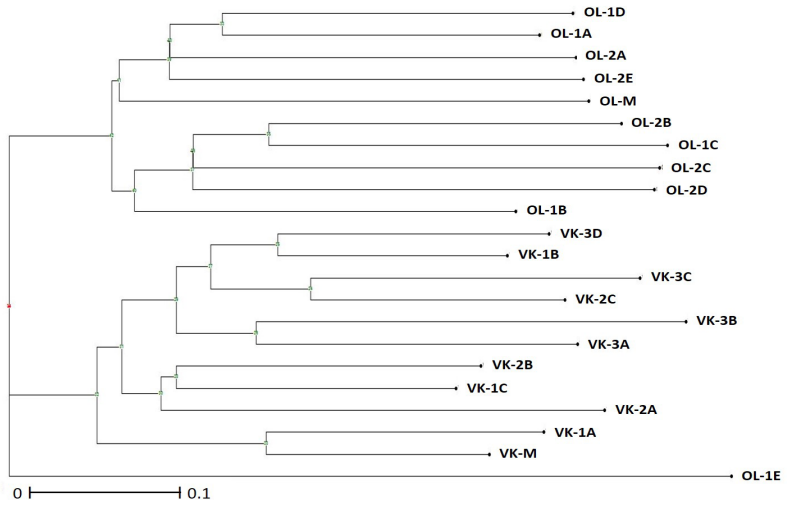

Figure 1: Dendrogram of zygotic and nucellar-originated embryos of polyembryonic genotypes along with mother plants based on SSR markers. VK-M Vellaikulamban mother plant, OL-M – Olour mother plant.

mother plant. In cluster III, the only embryo OL-1E was observed as a zygotic origin however this embryo was found distinct from its maternal Olour parent. Clusters I and II contained true-to-type nucellar embryos identical to their polyembryonic mother plants; however, a zygotic embryo (OL-1E) of polyembryonic Olour kernel in cluster III displayed a genetic distinctiveness.

In the present investigations, the embryos of polyembryonic Vellaikulamban and Olour kernels produce a zygotic embryo, or sometimes it may degenerate due to the dominance of the nucellar embryo's growth. According to the present findings, all individual Vellaikulamban kernel embryos had exhibited maternal origin except a few embryos. In Vellaikulamban, out of three kernels, only the first kernel had all embryos that resembled the maternal parent. In the second kernel, the 2nd and 3rd embryos were identified as zygotic by single primer MilIHR-26. In the third kernel, 3rd and 4th embryos were found to be zygotic by two primers, namely MilIHR18 and MilIHR26. In Olour, out of two kernels, the second kernel had embryos that resembled the maternal parent and in the first kernel, the 5th embryo was zygotic as shown by 6 primers. The identified primers, namely LMMA9, LMMA10, LMMA12, MilIHR29, MilIHR23, and MilIHR26 can be used for differentiating nucellar and zygotic embryos in Olour polyembryonic genotype. An attempt has been made for the first time to use individual kernel segments as samples for the identification of zygotic and nucellar embryos to overcome the ambiguity during the selection of seedlings of the selected polyembryonic genotypes. In Olour all six markers uniformly identified the embryo located farthest from the funicular point as zygotic when it was present. However, all the markers were not equally effective in discriminating zygotic and nucellar origin in the case of Vellaikulamban.

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