D-5351
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RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

Screening of Mango Landraces for Polyembryony and Confirmation of Seedling Origin using Microsatellite Markers

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10.18805/ag.D-5351

ABSTRACT

Background: The introduction of polyembryonic rootstocks in the area of propagation is of great importance since they produce one zygotic and several nucellar plantlets. Proper identification of sexual embryo from each hybrid seed is necessary in order to preserve only the nucellar seedlings, which would help to maintain the rootstock's genetic characteristics as well as to overcome the major constraints in the area of fruit breeding especially in hybridization programme by eliminating the nucellar ones to advanced generations. Contrasting reports exists regarding the vigour of zygotic seedlings of polyembryonic mango genotypes. It is necessary to identify/ distinguish the zygotic seedling from the nucellar population at an early stage, for which, microsatellite analysis could be a reliable tool.

Methods: The experiment was laid out in completely randomized design (CRD) with 20 treatments replicated thrice. The twenty local mango landraces from Thiruvananthapuram (Kerala) were screened for polyembryony and were geo-referenced. Germination studies were conducted. Microsatellite analysis of all the plantlets from two varieties which exhibited the highest polyembryony were done using SSR primers and their banding patterns were compared with those of their respective mother plants.

Result: Out of twenty mango varieties screened, seventeen were polyembryonic. Kappa Manga recorded the highest germination, germination index and seedling vigour index-I. Kotookonam Varikka recorded the highest polyembryony and followed by Kochu Kilichundan. Microsatellite analysis revealed that all the seedlings obtained from the respective stones of Kotookonam Varikka and Kochu Kilichundan had identical SSR profile to the mother plant, which indicated nucellar origin of seedlings having similar genetic composition to the mother plant.

Key word: Germination, Nucellar seedlings, SSRs, Vigour, Zygotic seedlings.

INTRODUCTION

Globally India leads in mango production as well as consumption. However the potential of mango for its commercial production has not been fully exploited. Selection of quality planting materials of improved varieties and adoption of scientific practices right from planting to harvest would serve to improve the productivity.

The mass multiplication of rootstocks from sexually developed seedlings, which are highly heterozygous has caused non uniformity in mango orchards (Srivastava *et al*., 1977). Ensuring uniform plant stand in commercial orchards is yet another pre-requisite for optimizing productivity, for which polyembryonic rootstocks can be utilized. In polyembryonic genotypes, the seedlings developed from nucellar embryos are clones of the mother plant (true-to-type), regardless of the pollen parent genotype. Hence they give more uniformity to the orchard, whereas this zygotic plantlets showing variability are mainly preferred for breeding programmes.

Identification of nucellar seedlings is usually done based on vigour. However, some studies suggest that zygotic seedlings need not always be less vigorous. In general the zygotic seedlings are smaller and weaker in nature than nucellar seedlings. Different methods *viz.,* flow cytometry, rootstock colour test, examination of morphological traits, biochemical markers and isoenzyme pattern analysis could not be employed commercially to discriminate both types due to their varying degrees of reliability.

Recently, various molecular markers have been adopted in many fruit crops for distinguishing the zygotic and nucellar seedlings (Rodriguez *et al*., 2004; Rao *et al*., 2008). Amongst various marker systems, the simple sequence repeats (SSRs) are very quick and more reliable to discriminate the zygotic and nucellar plantlets from both selfing and interspecific crosses (Ruiz *et al*., 2000).

Though, India is indigenous to some of the polyembryonic mango varieties, little work has been done on their nursery evaluation as well as on identification of DNA marker (s) for differentiating nucellar and zygotic seedlings, for identification of polymorphic SSR markers

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How to cite this article: Reshma, U.R. and Simi, S. (2021). Screening of Mango Landraces for Polyembryony and Confirmation of Seedling Origin using Microsatellite Markers. Agricultural Science Digest. DOI: 10.18805/ag.D-5351.

Submitted: 19-04-2021 **Accepted:** 02-07-2021 **Online:** 16-09-2021

among parental mango genotypes and their validation on hybrid population. Hence an attempt has been made to assess the germination behaviour and to screen the local mango varieties/ collections for the extent of polyembryony.

MATERIALS AND METHODS

The present investigation was carried out during 2018-19 at Department of Fruit Science, College of Agriculture, Vellayani, Thiruvananthapuram (Kerala). The experiment was laid out in completely randomized design (CRD) with 20 treatments replicated thrice. Twenty local mango varieties/ collections from different parts of Thiruvananthapuram district of Kerala were screened for polyembryony. These mother trees were geo-referenced. Microsatellite analysis of all the plantlets from two varieties which exhibited the highest polyembryony were done using twenty SSR primers (Table 1) and their banding patterns were compared with those of their respective mother plants. The treatments were T_1: Kotookonam Varikka, T_2: Thali, T_3: Vellari, T_4: Kochu Kilichundan, T_5: Unda Varikka, T_6: Paiveli Local, T_7: Vazhapazhithi, T_8: Pandi Manga, T_9: Champa Varikka, T_{10}: Kili Manga, T_{11}: Perakka Manga, T_{12}: Sreekaryam Local, T_{13}: Mylapoo, T_{14}: Kasthuri, T_{15}: Attanari, T_{16}: Pakalkkuri Local, T_{17}: Kuttara Local, T_{18}: Vellari Varikka, T_{19}: Kappa Manga and T_{20}: Nattumavu, respectively.

Germination and growth characters

Observations were recorded on germination (%), number of plantlets produced per stone, percentage polyembryony, mean germination time, germination index and seedling vigour index I (on growth basis).

The per cent polyembryony was calculated as;

$$\text{Per cent polyembryony} = \frac{\text{Stones having multiple seedlings}}{\text{Total number of germinated stones}} \times 100$$

(Kumar, 2015)

Mean germination time = $\sum f.x/\sum f$; where f is the number of stones germinated on day x (Czabator, 1962).

Germination index was calculated at 60 DAS as follows;

$$\text{Germination index} = \frac{\text{Germination percentage}}{\text{Time taken for 50 per cent germination}}$$

(Kendrick and Frankland, 1969).

Seedling vigour index- I was calculated at 120 DAS using the formula given by Rao *et al*. (2006).

Seedling vigour index-1 = germination percentage (%) x [shoot length (cm) + root length (cm)]

The experimental data was subjected to statistical analysis as per the method suggested by Panse and Sukhatme (1967). Treatment means were separated using F test values at 5% level of significance.

Microsatellite analysis

Young, tender and fully expanded leaves from the mother trees of Kotookonam Varikka and Kochu Kilichundan as well as the plantlets from stones of these two were collected. The genomic DNA were extracted using the method described by Dellaporta *et al*. (1983). The quantity of DNA present in each sample was determined by reading the absorbance at 260 nm and 280 nm in a spectrophotometer. Quality was assessed by using gel electrophoresis with 5ìl of crude DNA sample on agarose gel (0.8%) and stained with ethidium bromide.

PCR analysis

PCR reactions were carried out in a 25 µl reaction mixture which consisted of 2 µl of genomic DNA (~25 ng/µl), 12.5 µl PCR Taq Mixture, 2.5 µl forward primer (1 µM), 2.5 µl reverse primer (1µM) and 5.5 µl autoclaved distilled water. Thirteen primer combinations were screened by PCR (Table 1). The amplified products were run along with marker (50 bp ladder) on 2% agarose gel using 1X TBE buffer and stained with ethidium bromide. The profile was visualized under UV (312 nm) trans-illuminator and documented in gel documentation system (Syngene G box documentation system). The documented SSR profiles were carefully examined for the polymorphism in banding pattern among the plantlets with their respective mother plant.

RESULTS AND DISCUSSION

Germination characters of mango genotypes

The germination percentage differed significantly among the genotypes (Table 2). The highest germination (73.33%) was recorded in Kappa Manga and the varieties Vellari Varikka, Nattumavu, Kuttara Local, Unda Varikka, Pandi Manga and Champa Vaikka were on par. The least germination (31.11%) was recorded in Kotookonam Varikka. The germination capacity of mango genotype appears to be related to its stone size. Larger the seeds, more efficient will be germination (Kumar *et al*., 2018). The Kappa Manga had more stone weight than others. Hence it recorded the highest germination.

Out of seventeen polyembryonic varieties, Kotookonam Varikka produced significantly more number of plantlets/ stone (5.00) and followed by Kochu Kilichundan (4.13) whereas Pandi Manga recorded the least (1.67) (Fig 1). The phenomenon of polyembryony was of genetic nature and the frequency varied according to varieties. The intensity of occurrence of multiple seedlings is directly proportional to the number of embryos. Here the different categories (such as one, two, three, four and five plantlets per stone) varied significantly between cultivars under study. The probable reason of variations in sprouts of polyembryonic varieties is the failure of few embryos to germinate due to the temporary aberrations of embryos, which might be mediated through various extraneous factors (Barbosa *et al*., 2009).

The highest polyembryony (65.13%) was recorded in Kotookonam Varikka. The varieties Kochu Kilichundan and Sreekaryom Local were on par. The least (20.97%) was noted in Pandi Manga. The aberrant results obtained for polyembryony in mango genotypes might be attributed to their genotypes and genotype-environment interactions (Kumar, 2015).

The earliest mean germination time (17.50 days) was in Vellari Varikka. The varieties Nattumavu, Kappa Manga, Kuttara Local and Pandi Manga were on par. The maximum mean germination time (33.40 days) was in Kotookonam Varikka. The germination capacity of mango genotypes appears to be related to its stone size. Larger the seeds, more efficient will be germination and faster will be the radicle emergence than the smaller ones (Kumar *et al*., 2018). The delay in seed germination of Kotookonam Varikka might be due to the presence of hard seed coat as well as the competition among the seedlings.

Table 1: List of microsatellite primers and their base sequences.

Primer name	Sequence (5′-3′)	Annealing temperature (°C)	Melting temperature (°C)	Allele size range (bp)
SSR- 16	F: GCTTTATCCACATCAATATCC	54	54	160-170
	R: TCCTACAATAACTTGCC			
SSR-19	F: AATTATCCTATCCCTCGTATC	54	54	135-145
	R: AGAAACATGATGTGAACC			
SSR-20	F: CGCTCTGTGAGAATCAAATGGT	58	58	295-310
	R:GGACTCTTATTAGCCAATGGGATG			
SSR-24	F: GATGAAACCAAAGAAGTCA	53	53	310-346
	R: CCAATAAGAACTCCAACC			
SSR-26	F: GCCCTTGCATAAGTTG	52	52	170-182
	R: TAAGTGATGCTGCTGGT			
SSR-52	F: AAAAACCTTACATAAGTGAATC	52	52	207-248
	R: CAGTTAACCTGTTACCTTTTT			
SSR-84	F: TCTATAAGTGCCCCCTCACG	54	58	200-260
	R: ACTGCCACCGTGGAAAGTAG			
SSR-85	F: GCTTGCTTCCAACTGAGACC	52	58	250-310
	R: GCAAAATGCTCGGAGAAGAC			
SSR-89	F: CGCCGAGCCTATAACCTCTA	54	55	110-140
	R: ATCATGCCCTAAACGACGAC			
MNGSSR-14	F: TCATTAAGCTGTGGCAACCA	55	59	110-140
	R: CATTGCATAGATGTGGTCATT			
MiIIHR 10	F: CGATTCAAGACGGAAAGGAA	55	53	161-184
	R:TTCAAGCACAGACGACCAAC			
MiIIHR 11	F: CAGTGAAACCACCAGGTCAA	55	63.7	203-213
	R: TGGCCAGCTGATACCTTCTT			
MiIIHR 12	F: GCCCCATCAATACGATTGTC	55	53	153-187
	R: ATTTCCCACCATTGTCGTTG			
MiIIHR 13	F: CCCAGTTCCAACATCATCAG	55	50	169-193
	R: TTCCTCTGGAAGAGGGAAGA			
MiIIHR 15	F: CTAACCATTCGGCATCCTCT	55	54	135-194
	R:TCTGTGATAGAATGGCAAAAGAA			
MiIIHR 21	F: TTTGGCTGGGTGATTTTAGC	55	53	230-262
	R: TTAATTGCAGGACTGGAGCA			
MiIIHR 23	F: TCTGACCCAACAAAGAACCA	55	52	127-148
	R: TCCTCCTCGTCCTCATCATC			
MiIIHR 24	F: GCTCAACGAACCCAACTGAT	55	52	237-260
	R: TCCAGCATTCAATGAAGAAGTT			
MiIIHR 31	F: TTCTGTTAGTGGCGGTGTTG	55	52	210-229
	R: CACCTCCTCCTCCTCCTCTT			
MiIIHR 34	F: CTGAGTTTGGCAAGGGAGAG	55	53	222-244
	R: TTGATCCTTCACCACCATCA			

Table 2: Germination behaviour of different polyembryonic and monoembryonic mango genotypes.

Treatments	Germination (%)	Number of plantlets produced per stone	Polyembryony (%)	Mean germination time (days)	Germination index	Seedling vigour index-I(growth basis)
T_1	31.11	5.00	65.13	33.40	0.70	1029.01
T_2	46.67	2.27	29.57	29.63	1.27	1528.58
T_3	44.44	3.67	46.90	29.27	0.99	1283.70
T_4	33.33	4.13	63.62	30.17	0.76	910.18
T_5	60.00	2.27	23.12	28.57	1.66	2001.02
T_6	37.78	3.60	52.11	32.00	0.91	1174.07
T_7	55.55	2.20	30.97	27.23	1.52	1658.73
T_8	60.00	1.67	20.97	23.07	1.72	2233.78
T_9	60.00	2.27	28.06	28.40	1.53	1950.00
T_{10}	44.44	3.33	44.30	28.73	1.03	1365.16
T_{11}	46.67	3.27	41.79	30.57	1.12	1454.66
T_{12}	48.89	3.60	57.68	29.83	1.18	1643.55
T_{13}	40.00	3.80	56.30	32.73	0.96	932.57
T_{14}	51.11	2.33	33.88	32.80	1.21	1447.38
T_{15}	51.11	2.27	33.80	33.00	1.40	1799.25
T_{16}	51.11	2.33	41.75	28.50	1.35	1833.04
T_{17}	62.22	1.93	26.35	22.53	1.65	1580.91
T_{18}	68.89	-	-	17.50	2.15	2508.53
T_{19}	73.33	-	-	20.67	2.41	2795.20
T_{20}	66.67	-	-	18.90	2.27	2127.68
SE (m)	5.42	0.17	2.74	2.15	0.22	263.79
CD	15.55	0.49	7.90	6.17	0.62	756.74

-The monoembryonic varieties (T_{18}, T_{19} and T_{20}) were not included for statistical analysis.

The highest germination index (2.41) was in Kappa Manga. The varieties Nattumavu and Vellari Varikka were on par with Kappa Manga and the least germination index (0.70) was in Kotookonam Varikka. This significant variation might be due to the differential germination percentage recorded by different genotypes (Abirami *et al*., 2011).

The highest seedling vigour index-1 was noted in Kappa Manga (2795.20) and the varieties Vellari Varikka, Nattumavu and Pandi Manga were on par. The least vigour index (910.18) was recorded in Kochu Kilichundan. The higher result in Kappa Manga might be due to the vigorous seedling growth as vigour index-I is the product of germination percentage and seedling length. Besides, being a monoembryonic variety, presence of more endosperm tissue as well as higher stone weight might be a probable cause for more seedling growth compared to others (Rao and Reddy, 2005). The slow growth rate of seedlings in Kotookonam Varikka resulted in low seedling vigour index. In general, the probable reason for high and low growth potential of different genotypes might be due to their genetic constitution (Abirami *et al.,* 2011).

Molecular characterization of zygotic and nucellar seedlings

In polyembryonic varieties, there is one zygotic embryo (sexual) and several nucellar embryos which have the entire genetic constitution similar to that of mother tree (Sauco *et al.,* 2001). From the maternal nucellar tissue, the adventitious embryos are directly initiated, which surround the embryo sac containing a developing zygotic embryo (Aleza *et al.,* 2010). Hence, the identification of the zygotic embryo has great significance in mango. Furthermore, the nucellar embryos can be used to propagate disease free clonal rootstocks (Santos *et al.,* 2010). It is commonly believed that the most vigorous plantlets which arise from a polyembryonic seed are nucellar ones. Srivastava *et al.* (1988) ascribed that in polyembryonic mango seeds, the zygotic seedling might be the weakest and in lower proportion among the plantlet population on account of suppression of zygotic embryo with nucellar tissue or it perhaps degenerates due to the competition with nucellar plantlets.

There are many contradictory reports with respect to the identification of zygotic seedlings from polyembryonic mango genotypes. Cordeiro *et al*. (2006) revealed that the zygotic plantlet was most vigorous and later confirmed this with RAPD marker. In accordance with this result, Rocha *et al*. (2014) reported that the zygotic seedling need not be always weak. The zygotic plantlet will be vigorous in certain cases and grow healthy along with the nucellar plantlets.

Here the molecular characterization of Kotookonam Varikka and Kochu Kilichundan were done using SSR primers to compare the genomic DNA of mother tree with its offspring and SSR primers were evaluated for their ability

Kotookonam Varikka

Thali

Vellari

Kochu Kilichundan

Unda Varikka

Paiveli Local

Vazhapazhiti

Pandi Manga

Champa Varikka

Fig 1: continue....

Fig 1: continue....

Kili Manga

Peraykka Manga

Sreekaryom Local

Mylapoo

Kasthuri

Attanari

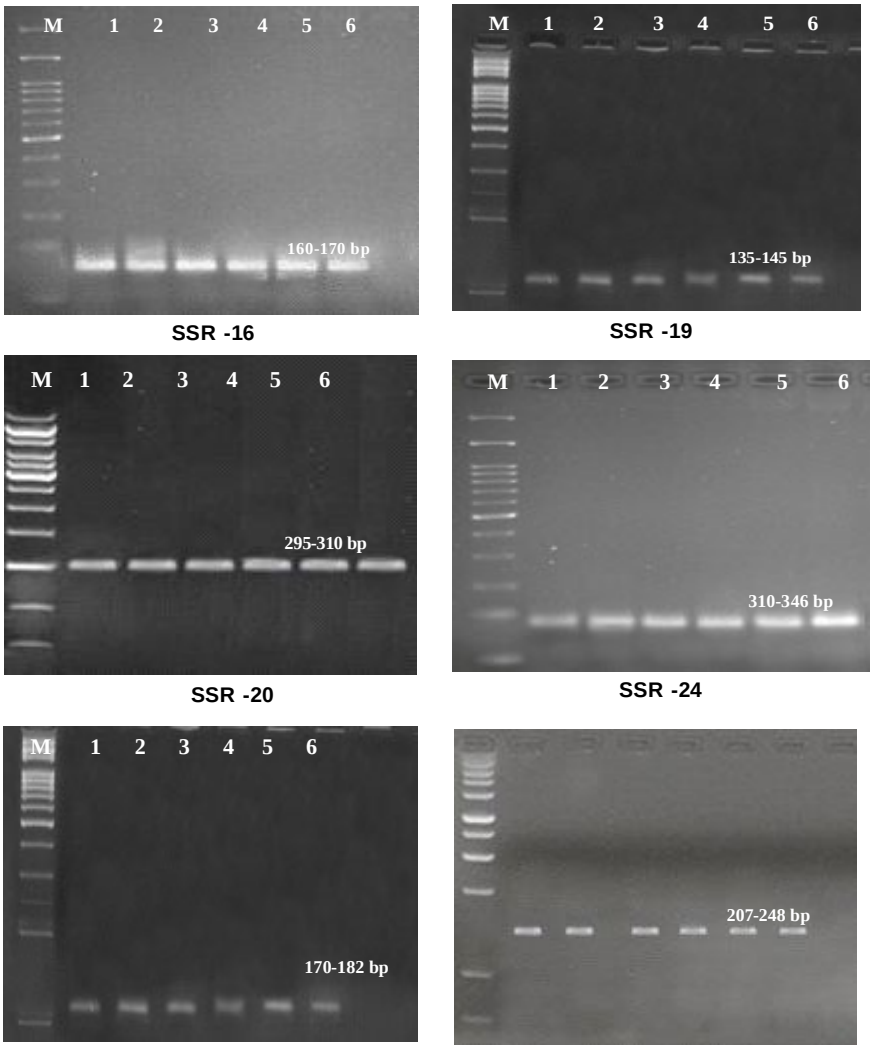
Pakalkkuri Local

Kuttara Local

Vellari Varikka

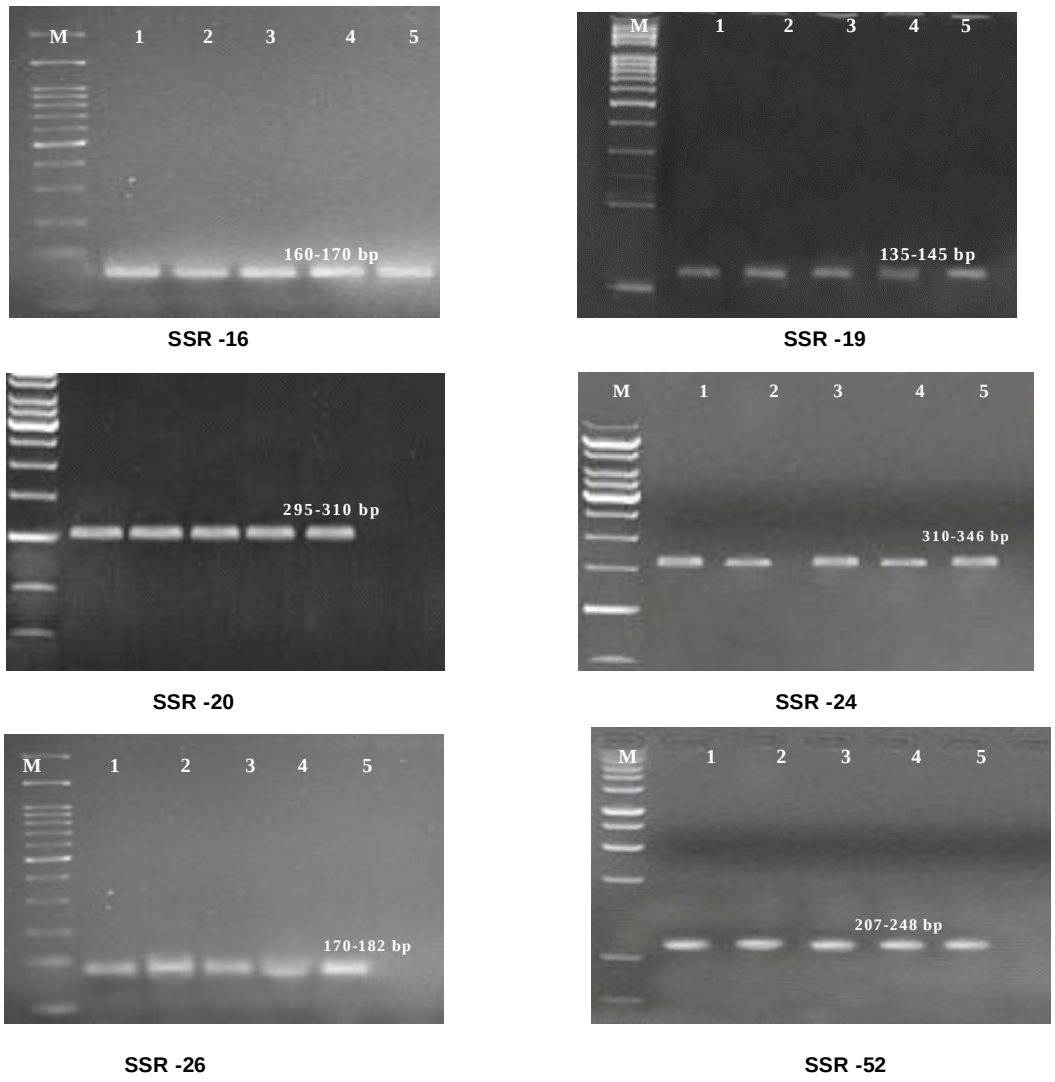
Kappa Manga **Nattumavu**

Fig 1: Extent of polyembryony.



1- Mother plant (Kotookonam Varikka), 2-6: Plantlets M: 100 bp ladder.

Fig 2: Amplification profiles of genomic DNA of plantlets obtained from var. Kotookonam Varikka and mother plant using SSR primers.



1- Mother plant (Kochu Kilichundan), 2-5: Plantlets M: 100 bp ladder.

Fig 3: Amplification profiles of genomic DNA of plantlets obtained from var. Kochu Kilichundan and mother plant using SSR primers.

to discriminate between zygotic and nucellar seedlings. The varieties were screened using 20 SSR primers (Table 1) which were reported in earlier works on mango (Begum *et al*., 2012; Sane *et al*. 2015) and found that the SSR primers uniformly amplified the DNA (Fig 2 and Fig 3).

It is evident that all the seedlings obtained from the respective stones had SSR profile identical to the mother plant. The identical banding pattern between multiple seedlings and mother plant indicated the nucellar origin of seedlings having the similar genetic composition (Dhillon *et al.,* 1993). Generally, the offspring from polyembryonic varieties, especially the nucellar ones are expected to be true to type and genetically identical to the mother plant (Shareefa *et al*. 2009). Any deviation from the banding pattern of mother plant, either presence or absence of any band could assure the zygotic origin of plantlet.

Most polyembryonic mango varieties occasionally produce morphologically off-type plants that presumptively are zygotic in origin (Schnell and Knight, 1992). From the present study, it can be presumed that the zygotic seedling has ceased growth and degenerated at very early stage of growth. Hence the identical SSR profiles of seedlings and mother plants ensure the nucellar origin of the seedlings. The nucellar ones could produce more uniform rootstocks and they could be used to generate homogeneous grafted plants. The result of the present investigation confirms that all vigorous seedlings of the polyembryonic mango varieties, Kotookonam Varikka and Kohcu Kilichundan can be used for clonal propagation to ensure homogeneity in orchards.

CONCLUSION

The variations in germination behaviour of mango genotypes mainly be attributed due to the variations in stone size and weight. Microsatellite analysis revealed the nucellar origin of plantlets and confirmed higher vigour of nucellar seedlings over zygotic seedling. The zygotic seedling might have

degenerated at very early stage of growth. The research works on genetic and morphological evaluation to characterize the nucellar and zygotic seedlings in polyembryonic mango varieties are meagre. More extensive works including other polyembryonic mango genotypes should be carried out to confirm the present results. Hence more evaluations are needed under different climatic conditions and with different varieties in order to prove this supposition beyond doubt.

ACKNOWLEDGEMENT

The authors are thankful to Dr. R. Beena, Assistant Professor, Department of Plant Physiology, College of Agriculture, Vellayani for her valuable suggestions during the critical stages of research work and Kerala Agricultural University for the award of fellowship.

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