Indian Journal of Agricultural Sciences **90** (11): 2101–7, November 2020/Article

SSR marker based differentiation of zygotic and nucellar seedlings in mango (*Mangifera indica*)

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Received: 30 October 2019; Accepted: 9 March 2020

ABSTRACT

Non-availability of standard clonal rootstocks in mango (*Mangifera indica* L.) is considered as an important hurdle, which has significant impact on orchard productivity. To obtain grafted mango plants, it is important to use polyembryonic rootstocks since they produce a zygotic and several nucellar plantlets from their seed, which are true-to-the type and also uniform. It is therefore imperative to device some reliable approach to ascertain the nucellar origin of seedlings to be used as rootstocks and thus culling out the variable zygotic seedlings in a polyembryonic genotypes for their use as uniform clonal rootstock. Differentiation of zygotic and nucellar seedlings using SSR markers in mango rootstock genotypes was undertaken during 2012-2015. Of the 42 SSRs used, 9 primer pairs (LMMA1, LMMA2, LMMA8, LMMA15, ESTD1, ESTD2, ESTD6, ESTD9 and ESTD10) were found to be informative, while 33 primer pairs were monomorphic. These nine primer pairs were used for differentiating zygotic and nucellar seedlings. In Olour rootstock, LMMA1, LMMA2, LMMA8, ESTD6 and ESTD10 primer pairs were informative and ascertained the zygotic and nucellar origin of seedlings. In Kurukkan rootstock, ESTD1, ESTD2, ESTD6 and ESTD9 primer pairs differentiated zygotic from nucellar seedlings. In 13-1 rootstock, LMMA8, LMMA15 and ESTD9 discriminated nucellars from zygotic seedlings. It is concluded that SSR markers were useful in differentiating the zygotic and nucellar seedlings in polyembryonic mango rootstocks and can be used in combinations to ascertain the origin of seedlings in polyembryonic mango rootstocks.

Key words: Mango, Nucellar, Polyembryony, SSR markers, Zygotic

Mango (*Mangifera indica* L.) cultivation in the country is hampered due to number of factors, *viz*. mango malformation disease, alternate bearing habit, physiological disorders, erratic bearing under climate change *etc*. However, non-availability of standard rootstocks in mango is considered as an important problem, which has significant impact on overall orchard productivity. Mango can be propagated by seeds or by grafting. For commercial purpose, grafting is the most appropriate method because it maintains the genetic uniformity of the propagated genotype. Several fruit species including mango has polyembryonic genotypes. Those mango varieties which are of Indian origin are chiefly monoembryonic. Many mango varieties of Indo-Chinese origin have polyembryonic seeds. To obtain grafted mango plants, it is important to use polyembryonic rootstocks since they produce a zygotic and several nucellar seedlings from single seed (Sturrock 1968). Polyembryony is characterized by the development of more than one embryo in the same seed, in which one zygotic and remaining are nucellar in origin. The nucellar plantlets maintain the genetics of the mother-plant and supposedly give more uniformity to the orchard and are preferred for grafting. In general, nurserymen use the most vigorous plantlets for grafting, believing that they are nucellar. However, orchard dis-uniformities in terms of canopy and yield is very common among mango trees in commercial orchards. It is therefore imperative to device some reliable tool to ascertain the nucellar origin of seedlings to be used as rootstocks and culling out zygotic counterparts in a polyembryonic genotypes for their use as rootstock. If a rootstock has more than 80% polyembryony, the possibility of obtaining nucellar plants increases and making it possible to have a uniform rootstock (Soares Filho *et al.* 2003; Santos *et al.* 2010).

Various methods have been attempted to discriminate the zygotic seedlings from nucellar's such as rootstock colour test (Furr and Reece 1946), flow cytometry (Tusa *et al.* 2002), thin layer chromatography (Tatum and Berry 1974), gas chromatography (Weinbaum *et al.* 1982), morphological

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traits (Bhat *et al.* 2010) and biochemical markers (Schnell and Knight 1992; Degani *et al.* 1993; Truscott *et al.* 1993) but none of these methods could be adopted at commercial level due to one or other problems. The use of molecular markers for differentiation of zygotic and nucellar embryos have also been tried in many fruit species (Rodríguez *et al.* 2004; Rao *et al*. 2008). Different DNA marker systems including RAPD (Bastianel *et al.* 1998; Rodriguez *et al.* 2004 & 2005; Srivastava *et al.* 2010), DAMD (Srivastava *et al.* 2010), ISSR (Shareefa *et al.* 2009; Srivastava *et al.* 2010) and SSR (Ruiz *et al.* 2000; Yildiz *et al.* 2013; Begum *et al.* 2013) were used in different fruit species for discrimination of zygotic and nucellar seedlings arising from single seed. The simple sequence repeats (SSRs) are molecular markers characterized by their highly polymorphic nature, abundance in the genome, high reproducibility and simple to use. These are ideal genetic markers for detecting differences between and within species (Farooq and Azam 2002). In the present study, a set of 42 SSRs have been used for differentiation of zygotic and nucellar seedings arising from single mango stone at an early stage of seedling development.

MATERIALS AND METHODS

The experiment was carried out at the Main Orchard of the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi situated at 28º40'N latitude and 77°12'E longitude with an altitude of 228.6 m above mean sea level during 2011-2015. New Delhi is located in Trans-Gangetic plains of agro-climatic zones of India. Climate is categorized as subtropical, semi-arid with cold winter and hot dry summer and it falls in the Agro-Eco-region-IV.

Germination and polyembryony

Polyembryonic mango rootstocks, namely, Olour and Kurukkan were collected from Mango Germplasm Block of ICAR-IARI, New Delhi, while stones of 13-1 rootstock and leaf samples of mother plants were collected from Horticulture Farm, M/s Reliance Industries, Jamnagar, Gujarat. Mature open-pollinated fruits were harvested and stones were extracted. The fully developed stones of polyembryonic mango genotypes were sown in earthen pots having sand, soil and FYM (1:1:1) under controlled polyhouse conditions (26± 2 ºC, 75-80% RH). Observations on germination were recorded after 30 days of sowing. The germination percentage was calculated by dividing the number of stones germinated by total number of stones sown multiplied by 100. The extent of polyembryony was calculated by dividing the number of stones having more than one seedling by total number of germinated stones multiplied by 100.

Isolation of DNA from mother plant and seedlings

Five gram of young, tender and fully expanded leaves from the mother tree and seedling plantlets arising from stones of polyembryonic mango rootstocks Olour, Kurukkan and 13-1 were collected, labeled wrapped in aluminum foil and put in a liquid nitrogen box for inactivation of enzymes. The leaves were washed and midribs and thick veins of the leaves were removed. Standard protocol of DNA isolation was carried out using CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Murray and Thompson (1980) with minor modifications (added 1% PVP w/v for removal of phenols). For purification of DNA, two µl RNase A was added per 200 µl DNA solution and incubated for 1 hr at 37°C and treated with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1).

Screening of SSR markers

Fourty-two primers including 16 microsatellites reported by Viruel *et al.* (2005), 15 by Schnell *et al.* (2005) and 11 EST derived SSRs were used for the investigation (Tables 1). The oligos were provided by the manufacturer in a lyophilized form. Based on the molecular weight of a given primer, a stock solution of 100 µM was prepared by adding the required amount of sterile double-distilled water and the stock was kept at -20°C overnight for proper dilution. PCR amplification was carried out with 50 ng of genomic DNA, 2 µl $MgCl_2$, 1U *Taq* DNA polymerase, 1x PCR buffer without $MgCl_2$, 0.5 µM of each of primers and 200 µM of dNTPs using Perkin Elmer 9600 thermocycler. PCR amplified products were resolved on submarine gel electrophoresis on 3.5% high resolution agarose gels along with 3µl Gene Ruler^{TM} (100 bp, Fermentas) at 5V/cm for 3 hr.

Statistical analysis

The experiment on differentiation of zygotic and

Table 1 Details of SSR primers (forward and reverse) and their base sequences.

	SSR	Sequence (5′-3′)
1	LMMA1	F: ATGGAGACTAGAATGTACAGAG
		R: ATTAAATCTCGTCCACAAGT
2	LMMA2	F: AAATAAGATGAAGCAACTAAAG
		R: TTAGTGATTTTGTATGTTCTTG
3	LMMA8	F: CATGGAGTTGTGATACCTAC
		R: CAGAGTTAGCCATATAGAGTG
4	LMMA15	F: AACTACTGTGGCTGACATAT
		R: CTGATTAACATAATGACCATCT
5	ESTD1	F: TGCTAATTTAGGCACTACCG
		R: ATCATTATCCACCTCCTCCT
6	ESTD2	F: TACCACTCGTAGCCTCAACT
		R: CCATTGTCGTTGTTGTTATG
7	ESTD6	F: CTGCAAATATCTCAGGCTTC
		R: CAGTGCGTTAGTTGTTGATG
8	ESTD9	F: GCTTTATCCACATCAATATCC
		R: TCGAACTAAAGAATTGGCAT
9	ESTD10	F: GATCTGACCCAACAAAGAAC
		R: ACGTAGATCTGCTTAACCCA

nucellar seedlings in three polyembryonic mango rootstocks was laid out in randomised block design (RBD). The data on germination and extent of polyembryony was analysed by using analysis of variance (ANOVA) using ExcelSTAT 2010. Valid conclusions were drawn only on significant differences between the treatment mean at 0.05 level of probability. Analysis of SSR molecular data for differentiating the origin of seedling was performed by comparing the banding pattern among mother tree and multiple seedlings. The bands generated by primers for each DNA sample was considered polymorphic if they were present or absent in at least one of the evaluated seedlings (Shareefa 2009). It is expected that nucellar seedlings have similar banding pattern as that of the mother tree and any variation in banding pattern noted due to presence as well as absence of some fragments from mother tree, is expected to be zygotic.

RESULTS AND DISCUSSION

Germination and polyembryony in mango rootstocks

Stone germination was significantly higher in Kurukkan and had non-significant differences with Olour. However, the minimum stone germination was observed in 13-1 rootstock. All the three rootstocks showed polyembryony and produced more than one seedling per stone. The extent of polyembryony was maximum in Kurukkan (66.0%) followed by Olour (36.5%) which had non-significant difference with 13-1 rootstock (30.0%) rootstocks. The average number of seedlings per stone was maximum in Kurukkan (2.03) followed by Olour (1.42) and 13-1 (1.22) (Fig 1). Variable results obtained for polyembryony percentage in mango rootstocks may be attributed to their genotypes and genotype and environment interactions. Several reports are available from different parts of the country for extent of polyembryony in different polyembryonic mango genotypes. Sane *et al.* (2015) recorded maximum polyembryony in Olour (84.4%) followed by Moreh (75.5%). Whereas, maximum polyembryony percentage was reported in Peach (338%) followed by EC 959862 (296%) and minimum in Kurukkan (138%) by Rao and Reddy (2005). Earlier, Srivastava *et al.* (1980) recorded maximum number of seedlings per stone in Vellaikolamban and Moovandan rootstock (1-7) and minimum in Mylepelian (1-3). Ochoa *et al.* (2012) observed 97 and 95% polyembryony in Manila and Ataulfo cultivars with an average 3.4 and 3.2 embryos per seed, respectively with more than 80% seeds were recorded with 2-4 embryos per seed.

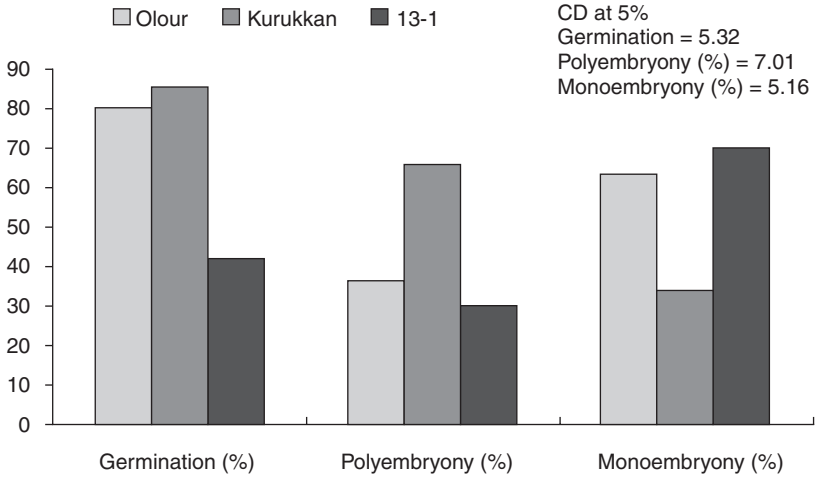

Fig 1 Germination, polyembryony and monoembryony percentage in mango rootstocks.

SSR markers based differentiation of zygotic and nucellar seedlings

In polyembryonic mango rootstocks, there is one sexual embryo and several nucellar embryos which have genetic constitution similar to the mother plant (Cordeiro *et al.* 2006). Adventitious embryos are initiated directly from the maternal nucellar tissue, which surround the embryo sac containing a developing zygotic embryo. Therefore, in mango, the identification of the zygotic embryo has great importance (Villegas and Andrade 2008). Initially, 42 SSR primers were used, out of which only 9 primer pairs, *viz*. LMMA1, LMMA2, LMMA8, LMMA15, ESTD1, ESTD2, ESTD6, ESTD9 and ESTD10 were polymorphic and found to be informative. These SSRs were further used for differentiating nucellar and zygotic seedlings obtained from stones of polyembryonic mango rootstocks.

Five SSR primers, namely, LMMA 1, LMMA 2, LMMA 8, ESTD 6 and ESTD 10 showed polymorphic pattern for Olour rootstock. SSR primer LMMA1 detected two common alleles in all the seedlings obtained from different stones and Olour mother plant. However, S_1 seedling of stone No. 1 had an additional allele. Similarly, S_2 seedling of stone No. 7 also had additional band. Whereas, S_2 seedling of stone No. 5, S_1 seedling of stone No. 6, S_1 seedling of stone No. 8 and S_2 seedling of stone No. 9 had only one allele and differed from the banding pattern of mother plant. Whereas, other seedlings obtained from these stone had similar banding pattern as it was in the mother plant. Primer LMMA1 clearly differentiated nucellar from zygotic origin seedlings arised from six out of nine stones (66.67%). The identical SSR profile of seedlings and mother plants ensure their nucellar origin. Whereas, deviation in SSR profile from mother plant indicates the zygotic origin of seedlings (Table 2; Fig 2a). Similarly, an additional allele in the range of 200 bp in S_1 seedling of stone No. 1, S_3 seedling of stone No. 2, S_2 seedling of stone No. 5, S_1 seedling of stone No. 6 and S_2 seedling of stone No. 9 was also detected by LMMA2 primer. It was interesting to note that the seedlings identified as zygotic by primer LMMA1 were also confirmed by this primer pair in stone No. 1, 5, 6, 8 and 9. The primer LMMA8 produced identical profile for majority of the seedlings arised from different stones and mother plant. However, an additional band was observed in S_1

Table 2 Zygotic and nucellar origin of seedlings of Olour differentiated by SSR primers

Primer	Stone 1					Stone 2			Stone 3		Stone 4		Stone 5		Stone 6		Stone 7		Stone 8		Stone 9		
	1	2	3	4	5	1	2	3	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3
LMMA 1	Z	N	N	N	N	N	N	N	N	N	N	N	N	Z	Z	N	N	Z	Z	N	N	Z	N
LMMA 2	Z	N	N	N	N	N	N	Z	N	N	N	N	N	Z	Z	N	N	N	Z	N	N	Z	N
LMMA 8	N	N	N	N	N	N	N	N	Z	N	Z	N	N	N	N	N	N	N	N	N	N	N	N
ESTD 6	N	N	N	N	N	N	N	N	Z	N	Z	N	N	N	N	N	N	N	N	N	N	N	N
ESTD 10	N	N	N	N	N	N	N	N	Z	N	Z	N	N	N	N	N	N	N	N	N	N	N	N

seedling of stone No. 3 and 4. Primer pairs, ESTD6 and ESTD10 also confirmed the polymorphism in S_1 seedlings of stone No. 3 and 4 (Table 2; Fig 2a).

In Kurukkan rootstock, four primers, *viz*. ESTD1, ESTD2, ESTD6 and ESTD9 showed polymorphism among seedlings obtained from different stones and mother tree. ESTD1 detected polymorphism in S_3 seedling of stone No. 4 and S_1 seedling of stone No. 6. The SSR profiles of other seedlings were identical to the mother tree. Interestingly, the polymorphic nature of S_3 seedling of stone No. 4 and S_1 seedling of stone No. 6 was also confirmed by the SSR markers ESTD2. In addition, ESTD2 also detected polymorphic S_2 seedling in stone No. 5. Furthermore, the zygotic origin of S_1 seedling of stone No. 6 was confirmed by ESTD6 and polymorphic nature of S_3 seedling of stone No. 4 was ascertained by ESTD9 (Table 3; Fig 2b).

Three primer pairs, *viz*. LMMA8, LMMA15 and ESTD9 were found to be informative in 13-1 mango rootstock. Primer pair LMMA8 showed polymorphic banding pattern indicating zygotic origin of S_3 seedling of stone No. 1, S_4 seedling of stone No. 2 and S_2 seedling of stone No. 5 (Fig 2c). LMMA15 also had polymorphic banding pattern in S_3 seedling of stone No. 1 and S_2 seedling of stone No. 5. This confirms the results obtained by using primer pair LMMA8. The ESTD9 primer pair showed different banding pattern from 13-1 mother tree in S_3 seedling of stone No. 12 and S_2 seedling of 13. Out of four seedlings obtained from stone No. 12, S_3 seedling showed single allele approximately 300

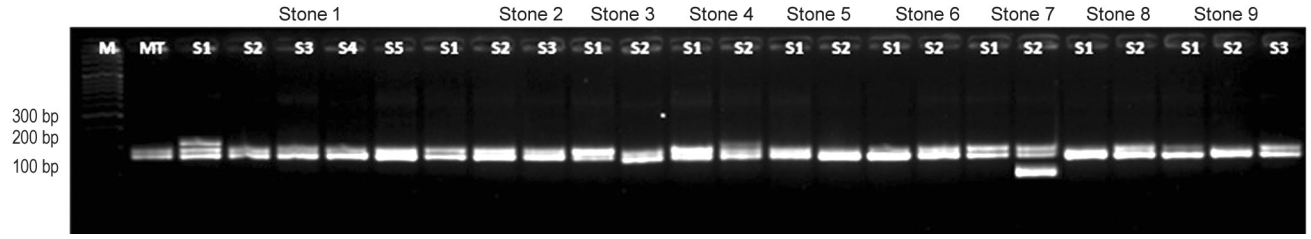

Fig. 2a LMMA1 profile of seedlings from nine different stones of polyembryonic Olour rootstock.

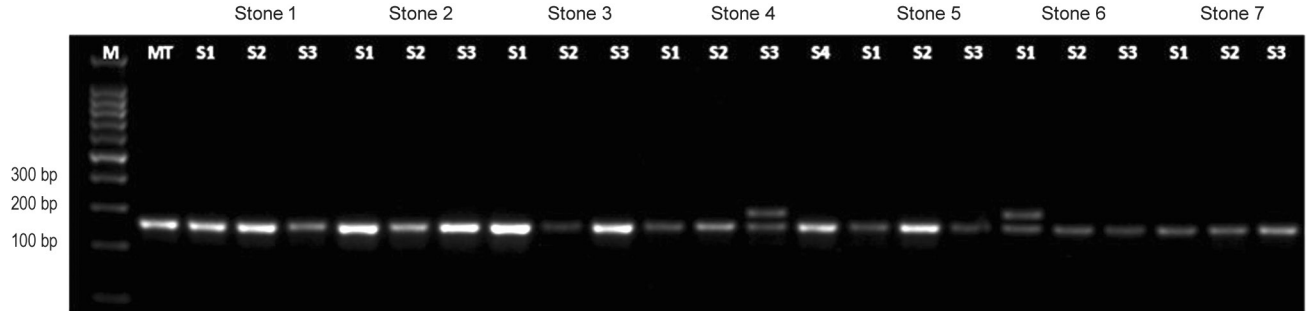

Fig. 2b ESTD1 profile of seedlings from seven different stones of polyembryonic Kurukkan rootstock.

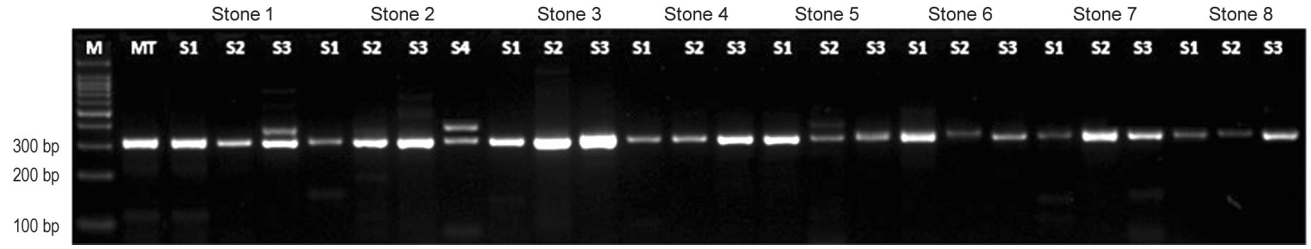

Fig. 2. LMMA8 profile of seedlings from eight different stones of polyembryonic 13-1 rootstock.

bp. However, in rest three seedlings and 13-1 mother tree, two alleles were observed. This suggest absence of band in S_3 seedling, which may be attributed to its homozygous condition of ESTD9 SSR, however, in other three seedlings identical to mother tree may be in heterozygous condition (Table 4).

In polyembryonic mango rootstocks, the SSR profile and polymorphism detected by specific primer could not ascertain the polymorphism in all seedlings obtained from all stones. However, in some cases the polymorphism was confirmed by more than one SSR primer. For example, in Olour, polymorphic S_1 seedling was confirmed by LMMA1 and LMMA2 primers. Similarly, polymorphic S_1 seedling of stone No. 3 and 4 was confirmed by LMMA8, ESTD6 and ESTD10 SSR primers. Out of seedlings arised from nine stones, LMMA1 and LMMA2 identified zygotic seedlings of five stone (66.67%). However, LMMA8, ESTD6 and ESTD 10 could differentiate zygotics from nucellars in 22.22% stones.

Similar trend was also observed in Kurukkan and 13-1 rootstocks and zygotic origin of particular seedling was confirmed by more than one primer pairs. In case of Kurukkan rootstock, ESTD2 primer pair differentiated zygotics from nucellars in 21.42% stones, while ESTD1 in 14.29% stones. In 13-1 rootstock, LMMA8 identified zygotics in 23.08% stones, LMMA15 and ESTD9 in 15.38% stones (Table 1b, c). This indicates that use of a combination of SSR primers were having better utility over use of single SSR primer pair. The identical banding pattern between multiple seedlings and mother tree indicated the nucellar origin of seedling having the similar genetic composition (Dhillon *et al.* 1993; Compos 1986).

This result may be attributed to the fact that zygotic seedlings from different stone may not have common male donor parent. Furthermore, the specific SSR may not be polymorphic among unknown male donor parents. Present results are in agreement to the findings of Rodriguez *et al.* (2004) who reported that OPI11 and P141 primers for differentiation of 12 zygotic seedlings from polyembryonic and monoembryonic seeds but no single primer could identify all the 12 zygotic seedlings. The differentiation of zygotic and nucellar seedlings by observing the polymorphism between zygotic and nucellar (mother type) was also reported by Cordeiro *et al.* (2006) and Ochoa *et al.* (2012) in mango using RAPD; Rocha *et al.* (2014) in mango using ISSR and Yildiz *et al.* (2013) in citrus using SSR. Similaly, Shareefa *et al.* (2009) reported 2 ISSRs primers out of 8 screened were polymorphic in Kinnow. The 6 primers were monomorphic among seedlings and mother plant. They obtained 5 ISSR markers showing polymorphic banding pattern in *Karna Khatta* and differentiated two zygotic seedlings. In the present study, the zygotic seedlings in all three polyembryonic mango rootstocks showed the distinct banding pattern from the mother plant, because of presence or absence of some fragments. This coincidence was also detected by Schnell and Knight (1992) using 5 isozymes to determine zygotic seedlings from 5 polyembryonic

Table 3 Nucellar and zygotic origin of seedlings of Kurukkan rootstock differentiated by SSR primers

Primer	Stone 1			Stone 2			Stone 3			Stone 4				Stone 5				Stone 6			Stone 7			Stone 8			Stone 9				Stone 10			Stone 11			Stone 12			Stone 13			Stone 14		
	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	4	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	3	4	1	2	3	4	1	2	3
ESTD 1	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
ESTD 2	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	Z	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	-	N	N	N	N	N	N
ESTD 6	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
ESTD 9	-	N	N	-	N	N	N	N	N	N	N	Z	N	N	-	N	N	N	N	N	N	N	N	N	N	N	-	N	N	-	N	N	-	-	N	N	-	N	N	-	N	N	N	N

Table 4 Zygotic and nucellar origin of seedlings of 13-1 differentiated by SSR primers

Primer	Stone 1			Stone 2				Stone 3			Stone 4			Stone 5			Stone 6			Stone 7			Stone 8			Stone 9				Stone 10			Stone 11				Stone 12				Stone 13	
	1	2	3	1	2	3	4	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	3	4	1	2	3	4	1	2
LMMA 8	N	N	Z	N	N	N	Z	N	N	N	N	N	N	N	Z	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
LMMA 15	N	N	Z	N	N	N	N	N	N	N	N	-	N	N	Z	N	N	N	N	-	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
ESTD 9	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Z	N	N	Z

N-Nucellar, Z- Zygotic

cultivars of mango. They observed that percentage of zygotic seedlings, which was varied from 0% (13-1) to 64% (Golek). They obtained 4% zygotics in Sabre, 24% in Turpentine and 36% in Madoe cultivars of mango and reported that 3, out of 8 rootstock mother trees of Turpentine were to be zygotic. The differentiation of zygotic seedlings from nucellars in *C. reshni* and *C. volkameriana* also reported the coinciding pattern (Rodriguez *et al.* 2005; Rodriguez *et al.* 2004). All the 8 markers were polymorphic in Attani (*Citrus rugulosa* Tan.). Out of 7, 4 seedlings were differentiated as zygotic which were obtained from 2 polyembryonic and 2 monoembryonic seeds (Shareefa *et al.* 2009). Truscott *et al.* (1993) identified zygotic seedlings in 5 polyembryonic mango cultivars using 3 isoenzyme systems and reported that percentage of zygotic seedlings ranged from 2 (Sabre) to 47% (Florigon). They obtained percentage of zygotic seedlings in Kensington, Julie and Peach as 12, 9 and 7 %, respectively.

Form the results of the present investigation, it is concluded that SSR markers were useful in differentiating the zygotic and nucellar seedlings in polyembryonic mango rootstocks. Nine primer pairs, *viz*. LMMA1, LMMA2, LMMA8, LMMA15, ESTD1, ESTD2, ESTD6, ESTD9 and ESTD10 were informative and thus can be used in combinations to ascertain the origin of seedlings in polyembryonic mango rootstocks.

ACKNOWLEDGEMENTS

Authors are thankful to ICAR-Indian Agricultural Research Institute, New Delhi for financial help under IARI Fellowship and University Grant Commission for Rajiv Gandhi National Fellowship during the Ph D degree of Shri Kamlesh Kumar.

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