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Development of Barcodes for Identification of Zygotic and Nucellar Seedlings in Polyembryonic Varieties of Mango (*Mangifera indica* L.)

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A B S T R A C T

Study on the seedling progenies of three polyembryonic varieties was carried out to differentiate zygotic and nucellar seedlings through molecular characterization. The fingerprinting showed variation across the varieties of selected seedling progenies. The variety Peach exhibited 100% zygotic seedlings among the varieties screened. The variety Nekkare was found to be 36.84% zygotic and minimum number of zygotic seedlings (10.52 %) was observed in Bappakkai. In breeding program as it is difficult to identify hybrid progenies of zygotic origin and identification of zygotic seedlings from nucellar is vital for a hybridization programme, wherein polyembryonic varieties are used as one of the parents. Hence, molecular markers are vital in identifying the seedlings in order to characterize the seedling progenies and parents by developing the barcodes of polyembryonic mango varieties to utilize in crop improvement.

Introduction

The mango (*Mangifera indica* L) regarded as one of the choicest fruits of the world, belongs to the family Anacardiaceae. It is considered to be the 'king of fruits', owing to its captivating flavour, delicious taste, irresistible sweetness and attractive aroma. It is believed to be originated in the Indo-Burma region (De Candolle, 1904 and Mukherjee, 1951). Its origin is traced back to 4000 years (De Candolle, 1884) and in India they are being associated with agriculture and civilization from time immemorial.

Traditional mango cultivars from a particular geographical region are genetically very similar (Ravishankar *et al.*, 2000). Depending on the mode of reproduction of seeds mango can be classified into two groups *viz*., monoembryonic and polyembryonic. Despite the intercrossability of mono and polyembryonic types and their wild occurrence, diverse genetic base is observed

for these types (Ravishankar *et al.,* 2004). The nucellar embryos can be used for raising 'true-to-type' seedlings and the uniformity of seedlings is beneficial. Polyembryony is one of the impediments since the outcome of hybridization is the development of zygotic recombinants. The identification of resultant hybrid progenies of zygotic origin from that of nucellar embryony is difficult from a cross when one of the parents or both the parents used is a polyembryonic variety. The number of seedlings that a polyembryonic variety generates varies from variety to variety and from region to region (Juliano, 1937).

Polyembryonic genotypes like 13-1 in mango possess most of superior traits such as dwarf stature and tolerance to salt (Schmutz and Ludders, 1993); Gomera-1 tolerant to salt stress (Martinez *et al.,* 1999); Nekkare and Olour tolerant to salt (Pandey *et al.,* 2014). In these cases polembryony is advantageous in clonal propagation, fixing of heterosis and restoration of vigour. However, they proved to be impediment in the breeding program as it is difficult to identify hybrid progenies of zygotic origin. Identification of zygotic seedlings from nucellar is vital for a hybridization programme, wherein polyembryonic varieties are used as one of the parents. Markers are vital in identifying the seedlings. In order to characterize the seedling progenies and parents an effort was made to develop the barcodes.

Materials and Methods

Fully matured and ripened fruits of the polyembryonic varieties namely, Nekkare, Bappakkai and Peach were collected from the mango field genebank of Indian Institute of Horticultural Research (IIHR) and stones were extracted from fully ripened fruits. Collected stones from fully ripen fruits were sown in polybags. Timely plant protection measures were taken for these half sib seedlings to maintain them in healthy condition. Recently matured leaf samples of both parents and offspring's were used for extracting DNA.

The genomic DNA was extracted from leaf samples by using CTAB (cetyl trimethylammonium bromide) method (Ravishankar *et al.,* 2000). PCR reaction was performed in a 10µl reaction volume containing 10X complete buffer, 25 mM $MgCl_2$, 1mM dNTP's, 0.3 µM primers, 0.5 U of Taq DNA polymerase (Homemade Taq) and 20ng template DNA in Biometra thermal cycler. Optimised reaction conditions for analysis were followed so as to get repeatable results. The amplified PCR products were then separated in 1.5% Agarose gel and viewed under UV light gel documentation system (UVi PRO, UK). The SSR profiling was carried out according to Ravishankar *et al.,* (2015). Samples were separated on an automatic 96-capillary automated DNA sequencer (ABI 3730 DNA Analyzer, Applied Biosystems, USA) at ICRISAT facility, Hyderabad, India. Generated raw data was analyzed and compiled using Peak Scanner v1.0 software (Applied Biosystems) to determine allele sizes. The results obtained were used for developing barcodes. Total of eight SSR markers developed by Ravishankar *et al.,* (2011) were used for developing barcodes. The details of the SSR markers used in this study are given in Table 1. Barcoding uses short genetic sequence from standard part of genome. It was done for both parents and half sibs using 'Barcode of life database' (BOLD, maintained by University of Guelph).

Results and Discussion

Eight SSR markers were used to develop the barcode. The details of the barcode generated for half sibs and their parents are presented in Figure 1. In the variety Peach none of the seedling progenies were observed to be similar to that of the maternal parent, whereas in the

variety Bappakkai 52.63 % progenies were similar to that of maternal parent and in the variety Nekkare 10.52 % progenies were similar to that of maternal parent.

DNA fingerprinting techniques using SSR widely used for cultivar identification in a wide range of species due to their high heritability and sufficient polymorphism to discriminate genotypes (Jeffreys *et al.,* 1985; Karp *et al.,* 1998). SSR markers are widely used for their multiallelic and codominant inheritance nature and the fact that they are highly suitable for high throughout PCR based platforms (Powel *et al.,* 1996; Zietkiewicz *et al.,* 1994). It was assumed that SSRs were primarily associated with noncoding DNA, but it has now become clear that they are also abundant in the single and low copy fraction of the genome (Yi *et al.,* 2006; Bindler *et al.,* 2007). In a highly heterozygous crop *viz*., mango where nomenclature ambiguity is one of the main hindrances in crop improvement (Vasugi *et al.,* 2013), DNA fingerprinting can be a very handy tool for individual identification of cultivars or rootstock for different horticultural purpose, such as breeder's right, identification of pollen parents and determination of genetic relatedness (Lavi *et al.,* 1993). The potential of SSR markers in fingerprinting is well established in mango (Viruel *et al.,* 2005; Shareefa, 2008). Validation of parentage by comparing the characteristics of the parents and hybrid progenies would help in the future breeding programmes. One of the very important conclusions that emerge out from this study also is which are all the varieties that can contribute to the progenies for certain desirable traits can be better explored for crop improvement programme.

In this study eight SSR markers were used to develop barcode for polyembryonic varieties and their half sibs. Half sibs of Peach exhibited 100% disimilarity from their maternal parent. Whereas in Bappakkai (10.52 %) progeny differed from their maternal pattern and 21.05 % of plantlets were considered doubtful as they differed with only one primer. In the variety Nekkare (36.84 %) differed from their maternal parent and 52.63 % were doubtful as they differed with one primer. This variation in different varieties might be due to heterozygosity existing in the variety and variation in per cent of nucellar seedlings. SSR allele size values generated in different laboratories are known to differ by 1 to 4 base pairs due to different analytical and rounding methods (This *et al.,* 2004). As such laboratory specific deviations tend to be systematic, they will cause a minor shift in the position of the size bars, but leave the overall barcode unchanged (Kanupriya *et al.,* 2011).

Table.1 Details of 8 SSR markers used in development of barcode

Locus	Repeat motif	H_O	H_e	PIC	F(Null)
MiIIHR17	(GT)13GAGT(GA)10	0.050	0.510	0.470	+0.8258
MiIIHR18	(GT)12	0.000	0.782	0.744	+1.0000
MiIIHR 23	(GA)17 GG(GA)6	0.017	0.728	0.693	+0.9541
MiIIHR 26	(GA)14 GGA(GAA)2	0.000	0.757	0.718	+1.0000
MiIIHR 30	(CT)13	0.044	0.762	0.713	+0.8910
MiIIHR 31	(GAC)6	0.024	0.885	0.862	+0.9469
MiIIHR 34	(GGT)9 (GAT)5	0.389	0.876	0.855	+0.3847
MiIIHR 36	(TC)17	0.000	0.845	0.818	+1.0000

(Source: Ravishankar *et al*., 2011)

H_O– Observed heterozygosity H_e – Expected heterozygosity PIC – Polymorphic Information Content

F(Null) – Frequency of null allele

Fig.1 Barcode developed for polyembryonic varieties and their half sibs [numericals (1,2,3) indicates individual stones and alphabets (a,b,c) indicates number of seedlings emerged from a single stone]

Peach (Maternal parent)		Bappakkai (Maternal parent)		Nekkare (Maternal parent)	
P1a		B1a		N1a	
P1b		B1b		N1b	
P2a		B1c		N2a	
P2b		B2a		N2b	
P3		B2b		N2c	
P4		B2c		N3a	
P5		B3a		N3b	
P6a		B3b		N3c	
P6b		B3c		N4a	
P7		B4a		N4b	
		B4b		N5a	
		B4c		N5b	
		B7a		N5c	
		B7b		N6a	
		B7c		N6b	
		B8a		N7a	
		B8b		N7b	
		B9a		N7c	
		B9b		N7d	

Polyembryony on mango is considered a genetic feature, although it is not yet known if it is a product of a recessive or dominant single gene (Sturrock, 1968; Aron *et al.*, 1998).

Polyembryonic seeds have one zygotic and from one to six nucellar plantlets depending on the variety. Zygotic plantlet in polyembryonic varieties was pointed out as the one which is the closest to the basal side of the seed and it degenerates or, do not develop well (Sachar and Chopra, 1957; Srivastava *et al.*, 1988). On the other hand, nucellar plantlets are those which develop very well and become the most vigorous in diameter and height. In this study opposite results were obtained.

On comparison of their allelic data with female parent showed that zygotic seedlings might be the vigorous one.

These results were in comparison with the findings of Cordeiro *et al.*, (2006).

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