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Research Paper

Application of Molecular Marker in Effective Management of Mandarin Orange Orchards of West Bengal

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A B S T R A C T

In West Bengal mandarin orange is grown in the Northern hilly region. Darjeeling Mandarin shows enormous commercial acceptance due to presence of high percentage of ascorbic acid with optimal ratio of sugar. The local orchards are affected by different biotic stresses. The sole morphological screening of seedling in nursery bed leads to the elimination of well performing genotypes. The occurrence of polyembryony and heterozygosity creates problem in breeding. The study shows recovery of zygotic and nucellar seedlings in 1:4 ratio. The management of these seed propagated outcrossed population requires application of advance marking system to detect the nature of off springs. In this paper the performance of three PCR based molecular markers RAPD, SSR, ISSR were evaluated showing efficiency in marking of pollen parents. The SSR primer CCSM13 with co-dominance and high PIC and MI gave best result. The maintenance of germplasm in perennial orchards require vast expenditure and skilled manpower. The inclusion of molecular marking in initial seedling stage in seedbed could aid in optimal management of orchard. In addition, the study revealed the scope of identification of elite genotypes to be included in Citrus hybridization programme and fixation of heterotic ability of the superior mandarin plant types.

***Key words*:** Darjeeling mandarin, Outcrossing, PCR marker, Nucellar, SSR, Polymorphic information content (PIC), Orchard management

Citrus reticulata is a perennial fruit-tree crop grown extensively in India. In West Bengal different Citrus species were grown including mandarin orange. The mandarin orchards are mainly located in the hilly North Bengal region producing very high quality of fruit called Darjeeling mandarin. The genotypes are subjected to different biotic stresses leading to dwindling of the orchards. The farmers of local orchards only depend on morphological evaluation of nursery seedlings for maintenance of orchards. The mandarin fruit crop in North Bengal reproduce through open pollination. In seedbed the off-type plants were rogued and an apparent homogeneous population of seedlings were raised for maintenance of the orchards. The sympatric inter-cross in open pollinated population may lead to production of economic hybrids [1]. The pollination behaviour of *Citrus reticulata* ensures natural hybridization but the chances of exploiting such germplasms reduces with off-type rouging [2]. These hybrids may assist in the rejuvenation of dwindling orchards. Mandarin is seed propagated and hardly carry virus and pathogens from the mother plants and naturally disease free with superiority over other planting material [3].

Information about pollen-mediated plant-to-plant gene flow from one citrus cultivar to other is therefore required to estimate the likelihood of incorporation of new genes in high-performing citrus varieties grown in orchards [4]. The inclusion of any agronomically significant character could also be possible from nearby plants due to high out-crossing rate and could facilitate orchard management ensuring genetic buffering. The well performing outcrossing genotypes could be included in hybridization programme [5]. PCR based molecular markers could be used for detection of outcrossing as they are reliable, heritable with ability to supersede morphological screening. In this paper mandarin seedlings were examined at morphological and molecular level to assess outcrossing.

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MATERIALS AND METHODS

Collection of fruit sample from selected plant and study of polyembryony

Mature fruits were collected and brought to the laboratory from a single mandarin (*Citrus reticulata)* plant from Lower Mirik region of Darjeeling district of West Bengal. The field characters were noted on spot and fruit quality parameters were assessed using [6] (Table 1). Seeds were separately extracted from individual plants following the procedure of [7]. The number of zygotic and nucellar embryos extracted from each fruit were recorded (Table 2).

Germination of seeds in soil mixture

After observation for primary records, the germinating seeds of one fruit was allowed to grow in aseptic conditions on a cotton bed for another 10 to 15 days, and then put into a sterile soil-sand-organic matter mixture (2:1:1) under

controlled conditions with high humidity for further growth of the seedlings, and were marked separately according to their origin. The growth pattern of different seedlings were carefully noted and recorded. When the seedlings were five-month-old with sufficient leaves were available for DNA extraction with good growth were selected. The progeny population of 22 seedlings originating from a single fruit were chosen. Out of 22 seedlings, 19 yielded good quality of DNA and were compared at molecular level for initial screening of type of DNA marker suitable for identification of outcrossing in mandarin orange.

Table 1 Field and physico-chemical characters of the plant selected from lower Mirik

Field character	Reading		
Age of plant	17		
Source	Seed		
Canopy structure	Open dome		
Bearing habit	Regular		
Average no. of fruits/plant	5000		
Harvesting time	Late december		
Physico-chemical	Mean	$Se_{m\pm}$	$CD_{(0.05)}$
Weight (g)	94.4	8.28	23.09
Circumference horizontal (cm)	18.02	0.79	2.23
Circumference vertical (cm)	16.10	1.01	2.82
Peel thickness	1.87	0.143	0.398
Number of locules	8.2	0.358	1.00
Number of seeds	9.6	1.64	4.58
Seed weight (g)	1.87	0.255	0.712
Pulp weight (g)	15.37	1.729	4.82
Juice content (ml)	52.40	5.436	15.16
TSS (°Brix)	10.32	0.371	1.034
Total sugar (%)	7.99	0.563	1.571
Reducing sugar (%)	3.34	0.252	0.704
Acid content (%)	0.484	0.058	0.162
Ascorbic acid (mg/100ml)	15.68	1.45	4.04

CTAB Leaf DNA Extraction Protocol by Doyle and Doyle (1990)

Genomic DNA was extracted from the tender leaves of the seedlings with the standard CTAB method of DNA extraction with minor modifications for *Citrus reticulata* [8]. The quantity and amount of DNA were determined as described by [9]. Standard CTAB method gave better result than extraction kit so that method was used for extraction of DNA.

Selection of suitable primers

DNA isolated from mandarin orange leaves and other *Citrus* plants were used for primer screening. Arbitrary decamer RAPD primer, Simple Sequence Repeat primer (SSR) and Inter-Simple Sequence Repeat primers (ISSR) were used after browsing through Google Scholar with a search including Citrus + Polyembryony + molecular marker and Citrus-Genomics and DNA marker. The screened primers of operon series or SSR and ISSR primers yielding more than one band and strong, intense, unambiguous and reproducible DNA fragment were selected for analysis of the progeny population (Table 3).

PCR procedure

Amplification using all the PCR primers was achieved by the protocol outlined by [10], with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1.5 unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X PCR buffer, and 0.5 µM primer of decamer primers (Bangalore Genei) in a total volume of 25 µL. DNA amplification using SSR and ISSR primers were carried out in 25 µl reactions containing 50 ng of template DNA, 5 µl of 0.2 mM total dNTPs, 3 µl of 0.5 µM primer, 2.5 µl of 10X PCR buffer, 1.5 mM of magnesium chloride and 1 unit of Taq polymerase (Bangalore Genei, India). The amplification was performed in a thermocycler (Gene Amp PCR System 9700, Applied BioSystems). The details of PCR protocol was given in (Table 4).

Electrophoresis of PCR product

Amplification fragments were separated on 1.5% (for RAPD), 2.0% (for SSR and ISSR) agarose (Merck-Genei) gels containing ethidium bromide (0.5 µg per ml of agarose) at 60 V for 6 hours in Tris Borate EDTA buffer. The gel was visualized and photographed under UV excitation using an electronic dual wave trans-illuminator system (Ultra.Lum Inc., USA).

RAPD, SSR and ISSR band scoring and cluster analysis

Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). Amplified bands from each primer were scored as present (1) or absent (0) for all the seedlings studied. The size of the fragments (molecular weight in base pairs) was estimated by using a 100 bp ladder marker (Bangalore Genei), which was run along with the amplified products.

Details of the software used for analysis

1. Excel version was used for calculating Mean value, coefficient of variation, Standard deviation, Polymorphic Information Content (PIC), Marker Index (MI) values.
2. TOTAL LAB software was used for calculating the fragment size of the generated amplicons.

RESULTS AND DISCUSSION

Studies on the morphological diversity of embryos

The nature and characteristics of polyembryony of a single mandarin orange (*Citrus reticulata*) plant was studied to understand the possible contribution of embryo source on phenotypic variation among the progeny seed population developed from a fruit of selected mandarin orange plant of Mirik, Darjeeling region. During fruit collection the leaves of the marked plant were collected in zip-bag and brought to the laboratory in pre-cooled box for DNA extraction. The mother plants DNA was extracted and included in molecular marking. The fruits were treated, cleaned and cut in laboratory to obtain the seeds. The seeds were kept in sterile Petri dish in moist cotton for germination. After 4-5 days the swollen seeds were selected for embryo extraction, all other seeds were maintained intact in cotton bed. Embryos were excised from the germinating seeds of these plants following the standardized procedure [11]. Some seeds were kept directly in plastic pots for direct soil germination for development of progeny population for molecular studies. Out of 21 progeny population 2 seedlings didn't performed well.

The zygotic embryo was present at the micropylar region holding the two original cotyledons of the seed. This embryo was usually bigger in size than the other embryos and also took the largest space in a seed. In most of the cases, rudimentary hypocotyls of the zygotic embryos became visible after removal of inner seed coat. Embryos originating from the nucellar tissues were tiny, green, and heart-shaped

and were crowded at the micropylar region or sometimes over the cotyledons of the zygotic embryos. Most of the seeds showed single normal zygotic embryo, but in a few seeds twin zygotic embryos was also observed. The percentage of zygotic embryo is only 18.41%. During hybridization the focus of the breeder lies on recovery of zygotic seedlings. In *Citrus reticulata* the percentage of nucellar seedling exceeds 80.58% showing difficulty in identification of zygotic progeny from the total seedling pool with simple optical observation (Table 2).

Table 2 The percentage of zygotic and nucellar embryos present in mandarin

Fruit number	Nucellar percentage	Zygotic percentage
1	80.39	19.61
2	86.12	13.88
3	76.19	23.81
4	80.77	19.23
5	77.78	22.22
6	85.00	15.00
7	85.08	14.92
8	84.13	15.87
9	80.39	19.61
10	80	20
SD	3.685362	3.342272
Mean	80.585	18.415
CV	4.573261	18.14973

In *Citrus* juvenility is another problem in assessing the performance of seedlings. The seedling takes 5-6 years to complete its juvenile period, then the anthesis, fertilization and fruit setting starts. Such a long vegetative stage hinders the assessment of the performance of the segregating progenies. The farmers discard the off-type seedling in nursery bed and that increases the chances of elimination of a quality genotype. In *Citrus* the polyembryony trait is quantitatively inherited though in mango the trait shows normal Mendelian inheritance with 3:1 segregation of nucellar and zygotic seedlings [12]. In *Citrus* the polyembryony trait shows complex inheritance and require proper detection of the true-hybrid in crop improvement programme. The detailed study of polyembryony also helps in the understanding of effect of out-crossing in a progeny population. In present study the ratio of nucellar: zygotic seedling shows a segregation ratio of 4:1. The understanding of self or cross compatibility along with estimation of outcrossing of elite genotypes assist a breeder in formulation of a proper breeding programme. This process also estimates inbreeding depression exerted by spurious genes and helps as an efficient orchard management tool.

Estimation of outcrossing by molecular marker

Plant genomic DNA was extracted using standard method of CTAB Plant DNA extraction. Quantification of DNA obtained by this procedure, was made by comparing the intensity of mandarin orange genomic DNA band with that of bacteriophage lambda DNA, Hind III cut samples (Bangalore Genei Ltd.). The concentration of DNA was also confirmed by spectrophotometric analysis as well. In this study an initial assessment was made among selection of primers for detection of outcrossing in *Citrus reticulata*. Only those bands showing consistent amplification were considered; smeared and weak. faint bands were excluded from the analysis.

Table 3 Details of the primers used in PCR analysis

Primer type	Locus code	Primer sequence		Repeat motif	T_m Value
SSR	CCSM 13	5'CTAGAGCCGAATTCACC3'(F)	5'AACATCTACCAAGACACC3' ®	AG	52°C(F), 52°C(R)
ISSR	HVH(CA)7T	5'CACACACACACACA3'	-	CA	52.1°C
ISSR	HVH(GT)7T	5'TGTGTGTGTGTGTG3'	-	GT	52.1°C
RAPD	OPAA 10	5' TGGTCGGGTG 3'	-		34°C

Table 4 Details of PCR Protocol for different primers

RAPD Cycling protocol, 45 cycles			SSR Cycling protocol, 36 cycles		ISSR Cycling protocol, 27 cycles	
Initial denaturation	94°C	30 seconds	94°C	4 minutes	94°C	4 minutes
Denaturation	92°C	30 seconds	92°C	1 minute	94°C	30 seconds
Annealing	38°C	30 seconds	51°C	1 minute	52°C	45 seconds
Extension	72°C	1 minute	72°C	2 minutes	72°C	2 minutes
Extended extension	72°C	5 minutes	72°C	10 minutes	72°C	7 minutes
Final extension	4°C	∞	4°C	∞	4°C	∞

Three types of markers were tested to identify the performance of molecular marker in outcrossing estimation in Citrus orchards. Arbitrary RAPD, Simple Sequence Repeat (SSR) and Inter Simple Sequence Repeat analysis (ISSR) all were able to yield good quality of bands. The random decamer primer OPAA 10 generated 13 polymorphic bands (amplicons), out of which 5 were unique. The size of the amplicons were ranging from 170 bp to 1150 bp. SSR primer pair CCSM 13 generated 10 polymorphic amplicons, out of which 5 were unique. The size of the amplicons were ranging from 220 bp to 957 bp (Table 5). Agarose gels for CCSM 13 showing amplifications of SSR of the 21 seedlings are presented including mother plant (M) in (Fig 1). Two Inter-Simple Sequence Repeat markers (ISSR) were used for identification of zygotic embryos. The products were separated on a 2% Agarose gel with TBE buffer. HVH(CA)7T generated 7 amplicons, out of which 3 were unique whereas HVH(GT)7T generated 11, out of which 2 were unique. The size of the amplicons were ranging from 150 bp to 1350 bp for (HVH(CA)7T) and 180 bp to 1345 bp for (HVH(GT)7T).

Simple Sequence Repeats (SSRs) genotyping is regarded to be a more reliable marker than the other PCR based marking because they are highly polymorphic and usually co-dominant, easy to use, evenly distributed in the genome, transferable between laboratories and not influenced by environmental condition [13]. Microsatellites are short sequence elements composed of tandem repeat units, 1-6 base pairs (bp) in length. These repeat sequences have shown to be highly polymorphic within species and applied in population genetics and genome mapping [14]. It has been used in the

genetic diversity studies of many plant species, genetic transformation of plants [15], and identification of zygotic plant [16]. The particular SSR (Simple Sequence Repeat) primer pairs developed by [17] for *Citrus* germplasm screening was used in this investigation to study their effectiveness in management of mandarin orange germplasm collected from the Mirik region of West Bengal. The unique amplicons suggest the possible identification of pollinators in open pollinated mandarin population.

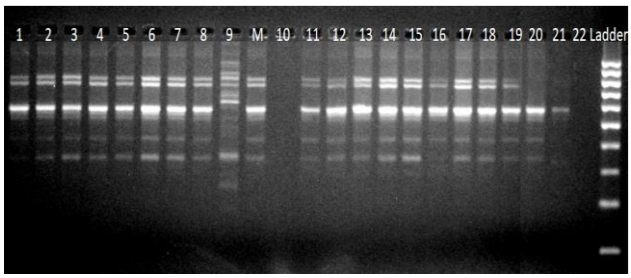

Fig 1 Polymorphism generated by SSR Primer CCSM13 with the segregated seedling progeny

ISSR marking system used degenerate primers to reveal a large number of fragments per PCR reaction and thus are able to efficiently distinguish among closely related individuals [18]. The two primers tested gave large number of bands but the gel resolution was not so high in 2% Agarose gel. The primer performance requires further evaluation. Polyacrylamide gel electrophoresis with silver staining may increase the number and resolution of bands. This work is initiated with two selected primers. Though the PCR protocol gave standard result but gel running require more specific standardization. This marking system is very useful for taxonomic studies of *Citrus* but its application for specific trait tagging is less [19]. Inclusion of more Inter-Simple Sequence Repeat Primers may prove beneficial for detection of outcrossing from open pollinated population. The amplicon profile of each seedling was compared with the mother to confirm the genetic architecture.

Introgression breeding in mandarin orange (*Citrus reticulata*) produced hybrid with morphometric parameter such as leaf apex playing pivotal role in identification of hybrids. The inclusion of SSR based DNA marking also confirmed the accuracy of hybrid identification on the basis of leaf apex. The result indicated visual selection and SSR marking could reduce cost, save time and increase accuracy of selection in Citrus breeding programs [20]. The Indian *Citrus* orchards are mostly old and their condition is deteriorating sharply for many reasons. The most important one being a disease complex, *Citrus* die back. Renovation of the orchard with new plantations requires disease free genotypes with proven desirable quality and high yielding ability [21]. Molecular marking could aid in selection of effective genotypes from an open pollinated population.

Table 5 The details of primer performance

Primer name	Number of loci	Polymorphic loci	Discriminatory amplicon	Polymorphic percentage (%)	PIC value	Molecular weight (bp)	Marker index
OPAA10	13	13	5	100	0.314	170 – 1150	4.082
CCSM13	8	8	3	100	0.580	220 – 957	4.608
HVH(CA)7T	7	5	3	71.43	0.415	150 – 1350	1.48
HVH(GT)7T	11	11	2	100	0.457	180 – 1345	5.027

Heterozygous DNA markers such as unlinked SSRs, amplified fragment length polymorphisms (AFLPs) or inter-simple sequence repeats (ISSRs) were able to detect hybrids. In polyembryonic seedlings many loci that are heterozygous in the female parent should be screened because, for each heterozygous locus, the probability of a selfed seedling having the same genotype as the mother is one in two. Molecular marker enabled identification of the apomictic genome region in grass family including *Paspalum simplex* species and sorghum, setaria, *Brachypodium* [22].

EST-SSR markers and transcriptome analysis clearly detected relative abundance of the common allele in sexual and apomictic samples in pre-anthesis stages, while it was up regulated in apomictic flowers at both anthesis and post anthesis stages. Apomixis-specific allelic variants were characterized by constitutive expression across all developmental stages. Furthermore, no obvious relationship was found between expression pattern of the selected apomixis-linked genes and their degeneration in hybrids [23]. Co-dominant marker provides best accuracy in hybrid identification but in its absence more dominant loci would be needed to achieve the same accuracy. The procedure thus become too laborious and costly to apply to seedlings from each of a large population of hybrids as would occur in the early stages of a breeding programme.

The use of DNA polymorphisms for the identification of outcrossing, cross-compatibility or hybrid detection is important in *Citrus* breeding programmes as it accelerates the process of progeny screening. Among DNA-based methods, Random Amplified Polymorphic DNA (RAPD) analysis is one of the most widely used for differentiating hybrids in *Citrus* breeding programs but is a dominant marker [24]. Both the ISSR primers were able to generate sufficient number of bands but being dominant marker efficacy is less compared to co-dominant markers. The single SSR primer used in this experiment proved beneficial in outcrossing estimation. In this study SSR primer, CCSM 13 gave best result in detection of outcrossing. As SSR markers are regarded as co-dominant, the polymorphism generated was regarded as most reliable and suitable to be applied in breeding programmes. The risk of outbreeding depression is overstated in fruit crops and empirical evidence supports larger impact of inbreeding depression on population [25]. In such case the accurate evaluation of outcross progeny is required before elimination of outcrossed genotypes. The superior performance of SSR marker indicates if the number of SSR primers increases the genetic heterozygosity of mandarin orchards could be accurately estimated leading to proper management of germplasms.

CONCLUSIONS

In this study the morphological differentiation of zygotic and nucellar embryos showed a 1:4 segregation. The thorough study of seedlings were not able to identify any morphological identifier. The selected molecular markers were able to screen the pollen parents. In this study DNA was extracted from only 5-month-old seedlings that indicates

possible application of this technique in low-cost orchard management with huge reduction in population size. An initial detection of pollen parent's contribution in open-pollinated population could surely minimize the expenditure of resource of the small growers and farmers which in long run could be utilized for the rejuvenation of the declining orchards. Additionally, estimation of outcrossing indirectly identifies the potential of genotypes to act as parent in *Citrus* hybridization program. PCR based molecular screening of genotypes can efficiently aid in orchard management by detecting true-to-the-type plants with high heterotic ability and novel genotypes to be used in orchard management programme.

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