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Application of simple sequence repeat markers for demarcation of *Citrus reticulata* nucellar and hybrid seedlings

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Abstract

The Polyembryony trait is a characteristic feature of the genus *Citrus*. This trait is regarded as a nuisance in hybridization but could be effectively utilized for Citrus propagation. The occurrence of nucellar embryony could be regarded as a replacement of costly tissue culture technique and identification of nucellar seedlings of *Citrus reticulata* may lead to proper management of the elite germplasms in mass scale propagation. Conventional techniques of 'Off type' rouging and other morphological identification techniques are not always correct. Molecular marker may be used for detection of nucellar seedlings. Simple sequence Repeat (SSR) markers were used for detection of nucellar seedlings. They are reliable markers and remain consistent under different environmental conditions. Four microsatellite primers with AG repeat CCSM13, CCSM17, CCSM18 and CCSM147 were able to demarcate the nucellar seedlings from the sexual ones. Three primers from TTA series TTA15, TTA27 and TTA33 were not able to discriminate seedlings. The four primers were able to discriminate the nucellar and zygotic seedlings developed from a local selection of an open pollinated population of *Citrus reticulata* of Darjeeling region of West Bengal. This experiment identified a few reliable markers useful for Citrus orchard management programme and could be applied for screening of asexual seedlings from an open population and also for controlled crossing.

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Introduction

Citrus reticulata is a very important fruit crop of India. In West Bengal the Eastern Himalayan region is considered as one of the centre of origin of citrus. The indo-china border is the birth place of several delicious types of *Citrus* with high palatability. Unfortunately today the major production of Citrus propagation is polyembryony, apomixes, long juvenility, self- incompatibility. Though polyembryony is regarded as a nuisance in conventional breeding but this phenomenon has serious importance in crop improvement as it can be utilized to fix heterosis of a hybrid in many crops and also utilized for recovery of true-to-the type progenies in the plants where prolong juvenility is the major constraint in breeding. In NEH region of India the farmers usually rogue off the dissimilar type off seedling to get a uniform population of citrus. The technique is not always effective as a good proportion of twin and triplets occur in the population (Mondal, 2006). Some unusual developments also take place in embryo development. A proper identification of nucellar and zygotic embryo is essential for routine propagation as well as for breeding programme.

Zygotic and nucellar embryos could be differentiated with the development of isozymes (Tusa *et al.* 2002, Soost *et al.* 1980). Although 18 enzymatic systems are known in *Citrus* still this analysis depends on time, tissue, enzyme type and age of plants. A shift to DNA based marker like Random Amplified Polymorphic DNA Markers (RAPD) have gained attention by some workers for its simplicity. This technique does not require any information of the target DNA and also gives enough polymorphism but are dominant in nature (Ferreira and Grattapaglia, 1995, Das *et al.* 2005). Microsatellites or simple sequence repeats are short sequence elements composed of tandem repeat units one to seven basepair in length (Tautz, 1989). These sequences are highly polymorphic and used in population genetics (Goldstein *et al.* 1999), and genome mapping (Weissenbach *et al.* 1992). SSR markers are becoming popular due to high polymorphism, co dominance, simplicity of analysis and repeatability (Thomas *et al.* 1994). The objective of this work is to differentiate the nucellar (asexual) and zygotic (sexual) seedlings of *Citrus reticulata* by PCR based SSR markers readily applicable for orchard management.

Material and method

Plant Material

The North Eastern Hilly region of India harbours very high yielding and good quality *Citrus reticulata* germplasms. The orchard suffers from decline syndrome due to unavailability of proper planting material. Some disease free, high quality planting materials are available but may not produce well after a long time due to accumulation of genetic load from unknown pollen parents. The fixed heterosis of a mother plant could be exploited for long if true to mother type plant is selected. A representative high yielding, late maturing plant of Mirik region of Darjeeling district of West Bengal was selected and marked for this study. This mandarin plant is important as it provides fruit when the other orchards become totally empty. Ten fruits were collected and some tender leaves were brought to laboratory for further analysis. Seeds collected from five representative fruits of each plant were surface sterilized with 0.1% mercuric chloride solution, placed between two layers of moist sterile cotton pad in Petri dishes, and incubated for 5 to 7 days at 35°C to germinate. Upon swelling of seeds, the germinating nucellar and zygotic embryos were identified following the procedure standardized by Tisserat (1985). Under aseptic conditions, the integument of the mature seed was carefully rolled away by making a longitudinal incision with a fine scalpel from the micropylar end. The germinating embryo holding the two original cotyledons and originating from the micropylar end was considered as the zygotic embryo. All other germinating embryos under the integument, each with two newly differentiating tiny cotyledons, were taken as nucellar embryos (Tisserat 1985). The germinating seeds were allowed to grow in aseptic conditions on a cotton bed for another 10 to 12 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 of six month old with sufficient leaves for DNA extraction was available twenty plants with good growth were selected.

DNA Extraction

Genomic DNA of the mother plant was extracted from tender leaves immediately after collection from the orchard. The DNA from seedlings was extracted after six months from the soft leaves of the seedlings using the Plant DNA CTAB Extraction procedure (Doyle and Doyle, 1987). The quantity and amount of DNA were determined as described by Kahangi *et al.* (2002) using standard DNA of uncut lambda bacteriophage in mini gel apparatus and spectrophotometric analysis.

Polymerase Chain Reaction

Amplification was achieved by the protocol outlined by Williams *et al.* (1990), with slight modifications. Ingredients of each reaction included template 25–30 ng DNA, 200 µM dNTPs each, 1unit Taq DNA polymerase, 2 mM $MgCl_2$, 10X PCR buffer, and 15 ng of both forward and reverse primers (EUROFINS, Germany) in a total volume of 25 µL. The amplification was performed in a thermal-cycler (Gene Amp PCR System 9700, Applied BioSystems). Total reaction consisted of 36 cycles of 1 minutes at 92°C, 1 minute at 36° C, 2 minutes at 72° C, with a final extension of 72°C for 10 minutes, followed by cooling at 20° C until recovery of the sample.

Electrophoresis

Amplified fragments were separated on 2% agarose (Sisco Research Laboratory) 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 transilluminator system (Ultra.Lum Inc., USA). Amplified fragments from all the primers were scored by the Total Lab gel documentation software (Ultra.Lum Inc., USA). The size of the amplicons (molecular weight in base pairs) was estimated by using a 100-bp ladder marker (Bangalore Genei), which was run along with the amplified products. The primers that could generate differential banding patterns of the seedlings of different origins (nucellar and zygotic) of a single fruit were noted. The bands were recorded as present (1) or absent (0) and complied into a two-way matrix (accession × marker). The molecular profile of seedlings was compared with the mother plant's profile for ascertaining the nature of the seedling.

Result and discussion

Seven SSR primers were tested for discrimination of hybrids from the nucellar seedlings of *Citrus reticulata* (Table I). Three SSR primers selected from TTA series TTA15, TTA 27 and TTA 33 were not able to discriminate seedlings and generated single amplicons after PCR reaction but the primers selected from CCSM series with AG repeat microsatellite loci (CCSM13, CCSM17, CCSM18 and CCSM 147) were able to differentiate sexual and asexual seedlings (Figure I). Ahmed *et al.* (2012) were able to identify hybrid with primers from TTA series. TTA15 proved useful for hybrid detection but in our experiment only a single amplicon was generated by all the seedlings without any noticeable polymorphism. Total 11 alleles were detected across loci. CCSM13 amplified 3 loci with wider amplicon range. CCSM 17 generated two locus of 100bp and 80bp. CCSM 18 generated two locus of 200 bp and 80 bp respectively. CCSM 147 amplified two locus of 100bp and 120 bp. In our study AG/TC motif gave variation in contrast to AT/TA motif. The seedlings showing similarity with the mother are regarded as nucellar but those showing difference were regarded as zygotic. The plant was selected from open pollinated population and the zygotic were regarded as off-type with different profile from the mother.

Gupta *et al.* (1996) reported the abundance of AT/TA repeat in plants but in *Citrus reticulata* the AG repeat shows more variation with respect to polyembryony trait. This study is in accordance with the analysis of Moriguchi *et al.*, 2003, He *et al.*, 2003b, Alghanim and Almirall, 2003, Ferguson *et al.*, 2004. Novelli *et*

al. (2006) created and evaluated four microsatellite markers for genetic variability study in *Citrus sinensis*. The four polymorphic microsatellite markers CCSM13, CCSM17, CCSM18 and CCSM147 were able to differentiate four cultivars of sweet orange.

Table 1. SSR primers used for PCR- Amplification of Genomic DNA extracted from seedlings of *Citrus reticulate*.

Marker	Forward Primer Sequence (5-3)F	Reverse Primer Sequence(5-3) R	Size of Amplicon (bp)	Observed Allele	PIC
TTA15	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	100	1	-
TTA27	GGATGAAAAATGCTCAAAATG	TAGTACCCACAGGGAAGAGAGC	110	1	-
TTA33	GGTACTGATAGTACTGGGGGG	GCTAATGCTAGGTCTTCGC	100	1	-
CCSM13	CTAGAGCCGAATTCACC	AACAGCTACCAAGACACC	950,280,80	3	.290
CCSM17	ACATGGACAGGACAACTAAG	GTTATGATACGTCTGTGTCC	80,100	2	.355
CCSM18	GTGATTGCTGGTGTCGTT	AACAGTTGATGAAGAGGAAG	80,220	2	.247
CCSM147	GCTATGTTATGATACGTCTG	AGACTCACGTAACCTACTTC	100,120	3	.345

The RAPD markers OPN12, OPA18, OPD08, OPA13, OPA07, OPM 06 were proved useful for detection of Polyembryony in *C. reticulata*. Decamer Primer OPH15, OPAT04 differentiates seedling in C. *aurantifolia* (Mondal & Saha, 2014). In an elaborate study with *Citrus reticulata* seedlings of Darjeeling location, four decamer primers OPA18, OPH11, OPB10, OPAA10 were proved very effective and grouped zygotic and nucellar seedlings into two distinct groups (Mondal *et al.* 2014).

Though the efficiency of RAPD primers in identification of nucellar seedlings was proved but very few investigations was done regarding the use of SSR or more reliable markers for identification of nucellar seedlings mainly in Citrus management programme of Indian subcontinent.

The primers from CCSM series were able to differentiate zygotic and nucellar seedlings as suggested by Novelli *et al* 2006. All the four primers were developed from a *Citrus sinensis* di or tri nucleotide enriched libraries. The origin of Citrus species suggests that *Citrus sinensis* is originated from a cross between *Citrus reticulata* and *Citrus grandis* so the primers qualifying well in *Citrus reticulata* plant types. In this study the influence of pollinator in embryo number was detected by SSR markers. This experiment was based on a progeny population developed from a open pollinated *Citrus reticulata* plant selected from Mirik, Darjeeling. The identified SSR markers require testing in other population to validate the reliability of the primers. The different population of Assam, Manipur, Mizoram, Arunachal Pradesh also produces good quality of mandarin and the identified primers could be applied on those population also.

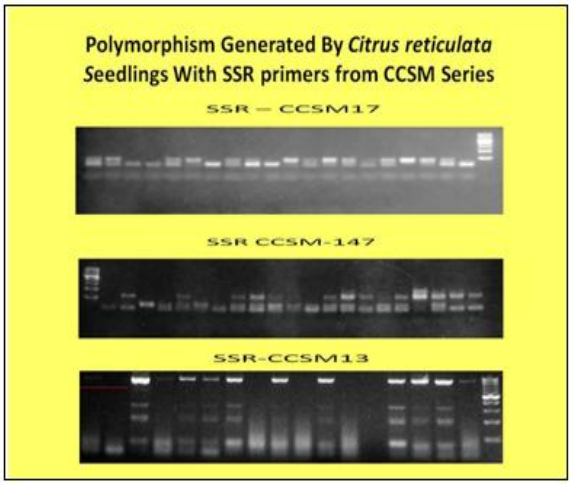

Fig. 1. Polymorphic patterns obtained from seedlings of the *Citrus reticulata* using the CCSM17,CCSM147 and CCSM13 locus.

This result suggests that strict analysis of various combinations of parents is essential for progress of citrus breeding programs. The high fidelity of SSR markers made them effective to identify nucellar seedlings as being genetically identical to their maternal parent and the finding may significantly contribute to orchard management programs by decreasing the space and time, and reducing genotypic duplication. Simple Sequence Repeats

(SSRs) have proven to be efficient genetic markers for comparative genetic mapping between *Citrus* species (Luro *et al*., 2008). However, relatively few citrus SSR markers have been published till date for identification of asexual (nucellar) seedlings from an open population. Cao *et al*. (2007) failed to detect differences among 16 *Satsuma mandarins* by SSR marking technique and therefore unable to detect the possible mutation origin of these variants. In our study we are able to differentiate seedlings developed from a single seed also confirming the role of SSR markers in embryonic discrimination. This attempt is a pioneering work for *Citrus* management of Eastern India and could be elaborated to overall germplasm improvement of India.

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References

Ahmed M, Javaid A, Rahman H, hussain SI, Ramzan A, Ghafoor A. 2012. Identification of Mandarin X Orange Hybrids using Simple-Sequence Repeat Markers. Journal of Agricultural Research **50(2),** 225-232.

Alghanim HJ, Almirall JR. 2003.Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. Analytical and Bioanalytical Chemistry **376(8),** 1225-33.

Q, Meng HJ, Wen XP, Yi HL, Deng XX. 2007. Genetic diversity of male sterile and low fertility germplasm of *Citrus* revealed using SSR markers. Chinese Journal of Agricultural Biotechnology **4,** 99-104.

Das A, Mandal B, Sarkar J and Chaudhuri S. 2004 RAPD profiling of some elite clones of Mandarin orange (*Citrus reticulata* Blanco) in the North Eastern Himalayan Region of India. Journal of Horticultural Science and Biotechnology **79**, 850–854.

Doyle JJ, Doyle JLA. 1987. Rapid DNA isolation method for small quantities of fresh tissues. Phytochemical Bulletin **19,** 11-15.

Ferguson ME, Burow MD, Schulze SR, Bramel PJ, Paterson AH, Kresovich S, Mitchell S. 2004. Microsatellite identification and characterization in peanut (*A. hypogaea* L.). Theoretical and Applied Genetics **108,** 1064-1070. http://dx.doi.org/10.1007/s00122-003-1535-2

Ferreira ME, Grattapaglia D. 1995. Introdução ao Uso de Marcadores RAPD e RFLP em Análise Genética. Embrapa - Cenargem, Brasília, 220 P.

Goldstein DB, Roemer GW, Smith DA, Reich DE, Bergman A, Wayne RK. (1999). The use of microsatellite variation to infer population structure and demographic history in a natural model system. Genetics **151,** 797–801.

Gupta PK, Balyan HS, Sharma PC, Ramesh B. 1996. Microsatellites in plants: A new class of molecular markers. Current Science **70,** 45-54.

He G, Meng R, Newman M, Gao G, Pittman RN, Prakash CS. 2003. Microsatellites as DNA markers in cultivated peanut (*Arachis hypogaea* L.). BMC Plant Biology **3,** 1-6. http://dx.doi.org/10.1186/1471-2229-3-3

Jannati M, Fotouhi R, Pourjan Abad A, Salehi Z. 2009. Genetic diversity analysis of Iranian citrus varieties usingmicro satellite (SSR) based markers. Journal of Horticulture and Forestry **1,** 120-125.

Kahangi EM, Lawton MA, Kumar CACY. 2002 RAPD profiling of some banana varieties selected by small scale farmers in Kenya.Journal of Horticultural Science and Biotechnology **77,** 393–398.

Luro FL, Costantino G, Terol J, Argout X,

Allario T, Wincker P, Talon M, Ollitrault P, Morillon R. 2008. Transferability of the EST-SSRs developed on Nules clementine (*Citrus clementina* Hort. ex Tan.) to other *Citrus* species and their effectiveness for genetic mapping. BMC Genomics **9,** 287.
http://dx.doi.org/10.1186/1471.2164.9.287

Mondal B. 2005, Studies on the molecular diversity of mandarin orange *(Citrus reticulata Blanco)* plant types collected from North Eastern Himalayan region. Ph.D Thesis, Bidhan Chandra Krishi Viswavidyalaya, India, 98-100 P.

Mondal B, Saha R. 2014. Identification OF zygotic and NUCELLAR SEEDLINGS of *Citrus reticulata* and *Citrus aurantifolia* using RAPD. International Journal of Advanced Biotechnology and Research **5(1),** 25-30.

Mondal B, Pal A, Saha R. 2014. Detection of zygotic embryos of *Citrus reticulate* by Random Amplified Polymorphic DNA technique. International Journal of Science and Research **3,**768- 773.

Moriguchi Y, Iwata H, Ujino-Ihara T, Yoshimura K, Taira H, Tsumura Y. 2003. Development and characterization of microsatellite markers for *Cryptomeria japonica* D. Don. Theoretical and Applied Genetics **106,** 751-58.

Novelli VM, Cristofani M, Souza A, Machado MA. 2006. Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* L. Osbeck). Genetics and Molecular Biology **29,** 90-96.

Soost RK, Williams TE, Torres AM. 1980. Identification of nucellar and zygotic seedlings of *Citrus* with leaf isozymes. Hort Science **15,** 728-729.

Tauz D. 1989 Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Research **17,** 6463–6471. 8.

Thomas VM, Clark CK, Schuldt CM. 1994. Effects of substituting feather meal for soybean meal on ruminal fiber fermentation and lamb and wool growth Journal of Animal Sciences **72(2),** 509-514.

Tisserat B. 1985. Embryogenesis, organogenesis and plant regeneration In: Dixon RA, ed. Plant Cell Culture, a practical approach, Vol.I. UK: Oxford IRL Press, Oxford, 79–104 P.

Tusa N, Abbate L, Ferrante S, Lucretti S, Scarano MT. 2002. Identification of zygotic and nucellar seedlings in *Citrus* interploid crosses by means of isozymes, flow cytometry and ISSR-PCR. Cell and Molecular Biology Letters **7,** 703-708.

Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vaysseix G, Lathrop MA. 1992. Second-generation linkage map of the human genome Nature **359,** 794-801.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research **18,** 6531–6535.