IDENTIFICATION OF MANDARIN X ORANGE HYBRIDS USING SIMPLE-SEQUENCE REPEAT MARKERS

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ABSTRACT

A study was conducted at Horticultural Research Institute, National Agricultural Research Centre, Islamabad, Pakistan during the year 2008-09 and 2009-10. The objective was to identify citrus hybrids using simple sequence repeat (SSR) markers. A total of 99 hybrid accessions from the crosses NARC 05-18 x Tarocco, NARC 05-17 x Sanguinello and Kinnow x Tarocco were tested using five SSR markers (TTA15, TTA27, TTA33, CCSM18 and CCSM147). Two SSR markers viz. TTA15 and CCSM147 were selected for hybrid identification. Twenty three hybrids from NARC 05-18 x Tarocco and five hybrids from NARC 05-17 x Sanguinello were identified using TTA15 whereas, 35 hybrids from Kinnow x Tarocco were identified using CCSM147 marker. The identified hybrids will be used in future citrus improvement programmes.

KEYWORDS: Mandrins; oranges; F_1 hybrids; molecular markers; Pakistan.

INTRODUCTION

Citrus is one of the world's important fruit crops widely grown at latitude 35^0 N-35°S (14). In Pakistan, it dominates with almost 31.0 percent of total fruit production (2). Citrus breeding has a number of serious limitations including its high genetic heterozygosity, longer juvenility, nucellar embryo interference/apomixes, self sterility or incompatibility of certain species, etc. (9).

Conventional breeding of citrus plants is slow and difficult, mainly because of the complex reproductive biology, involving polyploidy, high heterozygosity and single gene inheritance patterns in a few traits. Also, prolonged juvenile periods of seedling in the field makes citrus breeding very lengthy. Molecular based markers have been used to construct linkage maps, identify genotypes and distinguish hybrids from nucellar seedlings.

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An important constraint in citrus breeding programmes is apomixes. Apomictic embryos arising from the nucellar tissue give rise to seedlings which are genetically identical to seed parents thus yielding uniform offspring for propagation (14). In most of the crossing combinations, aimed at citrus improvement, it would not be possible to determine the genetic origin of seedlings from morphology. Identification of young seedlings is very essential for the plant breeders to avoid 5-10 years cost of growing and maintaining unwanted nucellar seedlings. Thus, it is mandatory to identify the zygotic seedlings while discarding the nucellar ones for smooth running of citrus breeding programmes. Genetic studies using microsatellite markers have increased rapidly because these are highly polymorphic, heterozygous conserved sequences which can be used as co-dominant markers (10).

In the past, various techniques were used to distinguish nucellar seedlings from sexual zygotic ones viz. colorimetric assays, infrared spectroscopy, enzymatic darkening of polyphenols, chromatography and isozyme pattern analysis (12). Until the discovery of DNA based markers, isozyme analysis remained the widely used technique for sorting out zygotic seedlings, since it is a fast and cheap methodology. However, variation depicted by isozyme banding pattern analysis suffers from limitation as only a few isozymic loci are available for marker analysis (14). The advent of PCR technique in the last decade has accelerated the development of various DNA based marker systems such as RAPD, AFLP and SSR. Ruiz *et al.* (14) described the use of sample sequence repeat (SSR) markers as an alternative method to distinguish sexual seedlings from nucellar ones. A lot of earlier research (1, 5, 6, 7, 18) on comparative efficiency of various PCR based DNA markers has proved beyond doubt that high level of polymorphism is always observed in case of SSR markers.

The present study was conducted to combine various fruit yielding and quality traits from divergent citrus groups into a single parent. Hence clear-cut demarcation for identification of zygotic seedlings remains a main hurdle in citrus breeding, indicating the need of a reliable and quick screening technique. Hence PCR-based SSR markers were employed for counter acting this short-fall.

MATERIALS AND METHODS

Plant material

Ninety nine F_1**s** derived from three crosses viz**.** NARC 05-18♀ × Tarocco♂, NARC 05-17♀ × Sanguinello♂ and Kinnow♀ × Tarocco♂ were used as plant material to identify the actual hybrids using molecular technique for further evaluation and multiplication of selected hybrids. The crosses were made at

Horticultural Research Institute, NARC, Islamabad, Pakistan during 2008-09 and 2009-10. The mature fruit of crosses were harvested in December, 2008 and seeds were germinated in incubator (20-25^{o}C). The germinated seedlings (F_1) were shifted to green house during March, 2009. The citrus hybrids were identified through molecular markers (SSR) technique from F_1 accessions during September to December, 2010. The breeding technique for the development of hybrids was used as reported by Robert and Cameron (13).

DNA extraction

The genomic DNA was extracted from leaves using the method of Coletta *et al.* (3). The quality and quantity of DNA were determined according to Valdenice *et al.* (17) described by Sambrook *et al.* (15). The DNA quantification was performed by using S30-Spectophotometer and rechecked through electrophoresis using standard λ DNA of known concentration through mini gel and optimum levels ranging from 50 to 200 ng were used for SSR amplifications.

Primers

Five SSR primers i.e. TTA15, TTA27, TTA33, CCSM18 and CCSM147 (Table 1) were used to study polymorphism between parent citrus varieties and hybrids (16). All primers were selected on the basis of previous published literature and among these two primers TTA15 and CCSM147 gave polymorphism for the parents used in hybrids which were selected for the identification of hybrids.

Polymerase chain reaction (PCR)

PCR was performed in volume of 20 µl with 2 µl of 10 × buffer (100mM Tris-HCL, pH 8.3, 500 mM KCL, 20Mm $MgCl_2$, 0.01% gelatin), 1.6 µl $MgCl_2$, 0.4 µl dNTP, 1 µl of primer, 0.2 µl Taq polymerase and 1µL DNA sample. The final volume was made upto 12.8 µl by adding distilled water. The reaction mixture was overlaid with mineral oil and the amplification was performed in thermocycler. The thermocycler was programmed for 36 cycles of 1 minutes at 92ºC, 1 minute at 36º C, 2 minutes at 72º C, with a final extension of 72ºC for 10 minutes, followed by cooling at 20º C until recovery of the sample.

Electrophoresis

PCR products were analyzed by agarose gel electrophoresis using 2 percent agarose gel containing ethidium bromide. Fragment sizes of PCR products

were estimated from the gel by comparison with 20-base pair DNA ladder marker. The bands were recorded as present (1) or absent (0) and complied into a two-way matrix (accession × marker).

RESULTS AND DISCUSSION

PCR analysis of parents and hybrids using SSR primers TTA27, TTA33 and CCSM18 produced no amplification or fragments with low resolution that could not be scored. DNA of parents and hybrids from the crosses NARC 05-18 x Tarocco and NARC 05-17 x Sanguinello was tested using SSR primer CCSM147 while parents and hybrids in case of cross Kinnow x Tarocco were tested using SSR primer TTA15 (Table 1).

Table 1. SSR primers used in the experiment.

Marker	Primer sequence (5-3) F	Primer sequence (5-3) R	Reference
TTA15	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC	Kijas *et al.*1997 (8)
TTA27	GGATGAAAAATGCTCAAAATG	TAGTACCCACAGGGAAGAGAGC	Kijas *et al.*1997 (8)
TTA33	GGTACTGATAGTACTGGGGGG	GCTAATGCTAGGTCTTCGC	Kijas *et al.*1997 (8)
CCSM18	GTGATTGCTGGTGTCGTT	AACAGTTGATGAAGAGGAAG	Valdenic *et al,* 2006 (17)
CCSM147	GCTATGTTATGATACGTCTG	AGACTCACGTAACCTACTTC	Valdenic *et al.* 2006 (17)

Since NARC 05-18, NARC 05-17 and Kinnow were used as female parents, accessions with banding pattern similar to Tarocco and Sanguinello were identified to be the hybrids. In case of cross NARC 05-18 x Tarocco, 31 accessions were analyzed using SSR marker CCSM147 (Fig. 1) and 23 hybrids (S1, S3, S5, S6, S7, S8, S10, S11, S12, S13, S14, S15, S16, S18, S19, S20, T1, T2, T3, T4, T5, T6 and T8) which had characteristic of both parental lines were identified. On testing six accessions from cross NARC 05-17 x Sanguinello using SSR marker CCSM147, five hybrids (A1, A2, A4, A5 and A6) were identified. PCR analysis of 62 accessions from Kinnow x Tarocco using SSR marker TTA15 (Fig. 2) identified 35 hybrids (KT2, KT11A, KT16, KT17, KT20, KT21, KT22, KT23A, KT29B, KT38A, KT54B, KT27, KT28, KT29, KT30, KT31, KT32, KT33, KT34, KT35, KT36, KT37, KT38, KT39, KT40, KT42, KT44, KT46, KT49B, KT50, KT51, KT52, KT57, KT66B, KT77A). Thus, a total of 63 hybrids were identified from 99 accessions (Table 2).

Table 2. Identification of hybrids among accessions obtained by different cross combinations using primers CCSM147 and TTA15.

Cross	Primer	Accessions	Hybrids
NARC 05-18xTarocco	CCSM147	31	23
NARC 05-17xSanguinello	CCSM147	6	5
Kinnow x Tarocco	TTA15	62	35

Simple-Sequence Repeat markers are considered to be one of the most efficient and informative tools to study genetic diversity in citrus (1, 6). SSR markers are also effectively used for identification of hybrids in citrus. Oliveira *et al.* (11) reported that combination of visual selection and SSR analysis for identification of hybrids derived from the cross of polyembryonic citrus cultivars can improve the accuracy of hybrids selection. Fu *et al.* (5)

performed SSR analysis of somatic hybrids between *Citrus sinensis* (navel orange) and *Clausena lansium* (sweet wampee). SSR analysis of seven randomly selected tetraploids and three triploids showed that they had specific fragments from both fusion parents, thereby confirming their hybridity. Ferrante *et al.* (4) determined the allelic configuration of eight new citrus tetraploid hybrids using SSR markers to observe capillary electrophoresis and PCR based dosage effects. Tetraploid hybrids were spontaneously obtained from different interploid crosses (2x × 4x) between diploid 'Femminello' lemon and allotetraploid somatic hybrid (2n = 4x = 36) 'Key' lime + 'Valencia' orange, and between diploid 'Wilking' and 'Fortune' mandarins and an autotetraploid 'Dancy' mandarin (2n = 4x = 36). The cytological mechanisms underlying the ploidy level of hybrids were determined using six SSR primers. The new genotypes with their improved genetic female background were considered valuable in citrus genetic improvement programme. Kamiri *et al.* (7) evaluated tetraploid inheritance in an interspecific somatic hybrid between mandarin and lemon through segregation studies using cytogenetic and SSR markers. SSR marker segregation was largely compatible with tetrasomic and inheritance intermediate between disomic and tetrasomic, with some evidence for preferential pairing of homoeologous chromosomes. Yaly *et al.* (18) studied the transferability of SSR primers developed for Pêra sweet orange (*Citrus sinensis* L. Osbeck) to determine the level of heterozygosity between citrus accessions and related genera. Twenty-four microsatellite loci were evaluated on 12 genotypes of citrus, poncirus, and an intergeneric hybrid. All analyzed markers were found to be transferable across all genotypes.

In present study 63 hybrids were identified among 99 accessions from three citrus cross combinations using SSR markers. The identified hybrids will be used in future citrus improvement programmes. Thus SSR markers can be used to identify citrus hybrids from parents showing polymorphism. It can improve the accuracy of selection and save time.

REFERENCES

1. Amara, M. H., M. K. Biswasa, Z. Zhangc and W. W. Guoa. 2011. Exploitation of SSR, SRAP and CAPS-SNP markers for genetic diversity of citrus germplasm collection. Scientia Horticulturae. 128(3): 220-227.
2. Anon. 2008-09. Agriculture Statistics of Pakistan. Ministry of Food, Agiculture and Livestock (Economic Wing), Government of Pakistan, Islamabad.

3. Coletta, F. H., M. Machado, M. Targon and J. Pompeu. 2000. The use of random amplified polymorphic DNA to evaluate the genetic variability of Ponkan mandarin (*Citrus reticulata* Blanco) accessions. Genet. Mol. Biol. 23(1): 169-172.
4. Ferrante, S. P., S. Lucretti, S. Reale, A. D. Patrizio, L. Abbate, N. Tusa and M. T. Scarano. 2010. Assessment of the origin of new citrus tetraploid hybrids (2n = 4x) by means of SSR markers and PCR based dosage effects. Euphytica. 173(2): 223-233
5. Fu, C. H., W. W. Guo, J. H. Liu and X. X. Deng. 2003. Regeneration of *Citrus sinensis* (+) *Clausena lansium* intergeneric triploid and tetraploid somatic hybrids and their identification by molecular markers. *In Vitro* Cellular & Developmental Biology Plant. 39(3): 360-364.
6. Jannati, M., R. Fotouhi, A. P. Abad and Z. Salehi. 2009. Genetic diversity analysis of Iranian citrus varieties using micro satellite (SSR) based markers. J. Hort. Forest. 1(7): 120-125.
7. Kamiri, M., M. Stift, I. Srairi, G. Costantino, A. E. Moussadik, A. Hmyene, F. Bakry, P. Ollitrault and Y. Froelicher. 2011. Evidence for non-disomic inheritance in a citrus interspecific tetraploid somatic hybrid between *C. reticulata* and *C. limon* using SSR markers and cytogenetic analysis. Plant Cell Reports. 30(8): 1415-1425.
8. Kijas, J. H. M., M. R. Thomas, J. C. S. Fowler and M. L. Roose. 1997. Integration of trinucleotide microsatellites into a linkage map of citrus. Theor. Appl. Genet. 94: 701-706.
9. Liu-Xu, X. Y. and X. X. Deng. 2007. Isolation of cytoplasts from Satsuma mandarin (*Citrus unshiu* Marc.) and production of alloplasmic hybrid calluses via cytoplast-protoplast fusion. Plant Cell Reports. 25(6): 533-539.
10. Novelli, V. M., M, Cristofani, A. A Souza and M. A. Machado. 2006. Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* L. Osbeck). Genet. Mol. Biol. 29(1): 90-96.
11. Oliveira, A. C., A. N. Garcia, M. Cristofani and M. A. Machado. 2002. Identification of citrus hybrids through the combination of leaf apex morphology and SSR markers. Euphytica. 128: 397-403.
12. Rao, M. N., J. R. Soneji, C. X. Chen, S. Huang and F. G. Gmitter. 2008. Characterization of zygotic and nucellar seedlings from sour orange-like citrus rootstock candidates using RAPD and EST-SSR markers. Tree Genetics Genomes. 4(1): 113-124.
13. Robert, K. S. and J. W. Cameron. 1975. Citrus. Advances in Fruit Breeding. p. 512-516.

14. Ruiz, C., M. P. Breto and M. J. Asins. 2000. A quick methodology to identify sexual seedlings in citrus breeding programs using SSR markers. Euphytica. 112(1): 89-94.
15. Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
16. Tan, M. L., J. K. Song and X. X. Deng. 2007. Production of two mandarin x trifoliate orange hybrid populations via embryo rescue with verification by SSR analysis. Euphytica. 157: 155.
17. Valdenice, M. N., M. Cristofani, A. A Souza and M. A. Machado. 2006. Development and characterization of polymorphic microsatellite markers for the sweet orange (*Citrus sinensis* L. Obsbeck). Genet. Mol. Biol. 29(1): 90-96.
18. Yaly, M. C., V. M. Novelli, M. Bastianel and M. A. Machado. 2011. Transferability and level of heterozygosity of microsatellite markers in citrus species. Plant Molecular Biology Reporter. 29(2): 418-423.