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Genetic Identification of Siam Madu and Satsuma Mandarin Somatic Hybrid Genotypes Using SSR Markers

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Abstract: Simple sequence repeat (SSR) analysis was used to identify the genetic status 23 citrus somatic hybrid genotypes from Siam Madu +Satsuma Mandarin and their similarity to the parental. Five SSR primer pairs could amplify polymorphic SSRs from all of these genotypes. A total of 17 alleles with an average of 3.4 alleles per locus were detected. The polymorphic information content (PIC) among genotypes varied from 0.076 (TC26 285) to 0.673 (AG14). Pairwise coefficients of genetic similarity between all genotypes ranged from 0.24 to 1.00. Unweighted pair-group method arithmetic average (UPGMA) analysis allocated the genotypes and their parents in 4 major clusters. Cluster I, consists of 6 genotypes and Siam Madu with 89% genetic similarity Cluster II consist of 14 genotypes with 91% genetic similarity. Cluster III consist of F17 and cluster IV consist of Satsuma Mandarin, with 58,6% and 24% genetic similarity, respectively. The genetic distance between all somatic hybrid genotypes with the parental Satsuma Mandarin is 0.60, except for F17 the genetic similarity is 0.34.
Keywords: Genetic, somatic hybrid, SSR markers, Siam Madu, Satsuma Mandarin.

INTRODUCTION

Citrus is an economically important fruit crop in Indonesia which is indicated by the fruit's import value that tends to increase every year along with the high consumer demand. However, the increasing imports of citrus fruit is not only driven by the needs of consumers, but also driven by the inability of local citrus to compete imports fruit in quality. It is needed for citrus breeding programs to develop new citrus cultivars from Indonesian local citrus with desirable characters to meet the increasing diverse demands of the market, such as easy peeling, seedless, good flavor and disease resistant. Actually, there are many kind of edible local citrus in Indonesia. The famous one is Siam Madu (*Citrus nobilis*) which has sweet taste character, high productivity and good adaptability in various agroecology zones [1]. However, Siam Madu also has undesirable characters that are seedy (15 – 20 seed/fruit) and hard peeling skin fruit. In order to decrease citrus fruit import rate, effort on improvement of Siam Madu unfavorable characters was already done via somatic hybridization between Siam Madu with Satsuma Mandarin, which is famous with its seedless and easy peeling characters.

Satsuma mandarin (*Citrus unshiu* Marc) is naturally seedless, and it was demonstrated by Yamamoto *et al*. via sexual hybridization and repetitive backcrossing that its pollen sterility is a CMS type [2]. Conventional breeding for transferring the CMS character from Satsuma mandarin to seedy citrus cultivars may encounter difficulties such as nucellar polyembryony, long juvenility, and wide incompatibility besides the maternal inheritance of CMS. Nevertheless, it may be achieved through somatic cybridization using Satsuma mandarin as embryogenic suspension parent, since it has been reported that citrus cybrids usually possess mitochondrial DNA (mtDNA) from callus parent and nuclear background from the other [3-5].

Somatic hybridization via protoplast fusion enables recombination of nuclear and cytoplasmic genetic information of both parents as well as provides an alternative strategy to bypass several difficulties, such as wide incompatibility, nucellar polyembryony and pollen/ovule sterility encountered in citrus conventional breeding. Nowadays, it has been a successful and promising biotechnology for citrus rootstock improvement as well as scion breeding [3, 6-9].

Somatic hybridization between Siam Madu + Satsuma Mandarin had succeed and resulted hybrid

genotypes which were planted in pots. Even though they grew well, we are still not aware of their genetic identities which is essential for genotypes selection based on desirable characters. Genetic identification via molecular method becomes necessary to be done in early step in somatic hybridization citrus breeding program. Several molecular methods are broadly used to understand citrus fingerprinting, such as RAPD (Random Amplified Polymorphic DNA) and RFLP (Restriction Fragment Length Polymorphism) [4, 10]. Nevertheless, in developing efficient and simple marker, Simple Sequence Repeat Marker (SSR) seem to be a suitable alternative technique based on their hyper-variability, co-dominance and high reproducibility and have been widely used in genetic studies of different plant species such as *Ziziphus jujuba* Mill. [11], *Rosa hybrid* L. [12] , *Pyrus communis* L. [13] and *Prunus tomentosa* Thunb. [14].

Many studies have shown that SSR markers were already used to analyze genetic character citrus accesions and hybridzation [15] such as analyze genetic diversity of Iranian orange (*Citrus sinensis* (L.) Osbeck) and Mandarin (*Citrus reticulata* Blanco) [16], and useful for molecular characterization of intergeneric somatic hybridization between tangerine + citrange . Kijas already developed 14 citrus SSR primers based on PCR technique including TAA15 and TAA41 loci [17] . Barkley [18] also evolved 11 citrus SSR primer that consisted of CAT01 and AG14. These primers are freely available for citrus.This study was order to identified genetic siam madu + satsuma mandarin somatic hybrid genotypes using SSR markers.

MATERIALS AND METHODS

Plant materials

Twenty three plants of somatic hybrid from Siam Madu+ Satsuma Mandarin and their related parents (Table 1) were collected from the Tlekung Experimental Orchard of Indonesian Citrus and Subtropical Fruit Research Institution (ICSFRI), Batu, East Java, Indonesia.

Table 1. Sample and accessions code of somatic hybrid plants from Siam Madu + Satsuma Mandarin

Sample code	Accessions code	Sample code	Accessions code
1	FS29 (I)	13	FS I
2	FS 33	14	FS (II)
3	FSI (4)	15	FS 25
4	FS 17	16	FS 2 21
5	FS 29 (I)	17	FS 18
6	FS I 19	18	FS 29 (I) 1.1
7	FS I 8	19	FS I 3
8	FS 31	20	FS 29 (I) 1.2
9	FS I 6	21	FS I 3
10	FS I 23	22	FS 15
11	FS 13	23	FS I 22
12	FFS 12		

DNA isolation

Total genomic DNA was isolated from fresh leaves following the procedure previously described by Cheng *et al*. [19]. The quality of the DNA samples were checked in 0.8% agarose gel electrophoresis and visualized by Biodoc analyzer (Biometra).

SSR analysis

SSR amplification was performed as described by Biswas [20] with minor modifications. Amplification reactions were performed in 20 µl of Fast Start reaction mixture from Roche with 5 pmol of each primer (forward and reverse) (Table 2) and 50 ng template DNA. The amplification reaction procedure was as follows: after denaturation at 94 °C for 4min, the reaction mixture was subjected to amplification for 30 cycles consisting of 50 s at 94 °C, annealing at 51 °C for 1 min and a final extension at 72 ◦C for 10 min. The amplification products were separated by 3% agarose gel electrophoresis and visualized by a simplified silver staining method previously described by Xu *et al*. [21].

Table 2. Code and sequences of SSR primers used for pedigree and parental genome comparation

No.	Nama Primer	*Forward-Primer*	*Reverse-Primer*
1.	TAA15	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC
2.	TAA41	AGGTCTACATTGGCATTGTC	ACATGCAGTGCTATAATGAATG
3.	CAT01	GCTTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC
4.	AG14	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC
5.	TC26	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA

Data analysis

Polymorphism information content (PIC)

Polymorphism information content (PIC) values were calculated according to Smith *et al*. [22] using the following formula as follows:

$$PICi = 1 - \sum_{j=1}^{n} Pij^2$$

Where PICi is the polymorphic information content of a marker i; Pij is the frequency of allele for marker i and the summation extends over n patterns. PIC provides an estimate of the discriminatory power of a locus by taking into account, not only the number of alleles but also the relative frequencies of those alleles. PIC values vary from 0 (monomorphic) to 1 (very highly discriminative with many alleles in equal frequencies).

Genetic similarity (GS)

For the purpose of assessing genetic diversity leading to cluster analysis, the data in binary format (1, 0) were used to compute pair-wise similarity coefficients [23], utilizing the SIMQUAL (similarity for qualitative data) method in NTSYS-pc software.

Cluster analyses

A similarity coefficient matrix was used for cluster analysis following UPGMA (unweighted pair group method with arithmetic averages), which is one of the several SAHN (sequential, agglomerative, hierarchical, and nested) clustering methods that are available [24]. NTSYS-pc software was used for analysis and the resulting clusters were represented in the form of a dendrogram.

RESULT AND DISCUSSION

A total of twenty three plants somatic hybrid from Siam Madu + Satsuma Mandarin and their related parents were determined by 5 SSR markers; TAA15, TAA41, CAT01, AG14 and TC26. The number of alleles observed in the five microsatellite systems with their average band size, the allele frequencies and PIC values in 23 somatic hybrid plants from Siam Madu + Satsuma Mandarin are given in Table 3.

Table 3. Polymorphism of five microsatellite (PICi) loci in somatic hybrid plants from Siam Madu + Satsuma Mandarin

Marker	No. of Alleles	Allele size (bp)	Allele frequencies	PICi
TAA15	5	150 167 183 192 200	0.038 0.038 0.538 0.038 0.346	0.584
TAA41	3	142 158 175	0.480 0.060 0.450	0.562
CAT01	3	133 150 158	0.500 0.480 0.020	0.519
AG14	4	150 160 165 180	0.320 0.010 0.320 0.320	0.673
TC26	2	153 167	0.960 0.040	0.076

The most allele number was revealed by TAA15 marker with 5 alleles, ranging in size from 150 to 200 bp. The TAA41 and CAT01 microsatellite were also polymorphic, with 3 alleles with sizes 142–175 bp and 133–158 bp, respectively. Four alleles with sizes ranging from 150–180 bp were observed on AG14 markers and two alleles with size from 153–167 bp were detected on TC26 marker. There allele frequencies were vary among markers between 0.010 – 0.960. Microsatellites containing tandem TAA repeats have been useful markers in several species [25]. This study proves that TAA15 is a huge potential marker for citrus genetic identification which could differentiate many alleles better than the rest of the marker tested. Figure 1 showed an example of DNA profiles amplified by TAA15 marker with 5 differences alleles among somatic hybrid genotypes and the parents.

The polymorphic information content (PIC) is generally used to evaluate a marker system for its ability to detect high levels of DNA polymorphism in an analysis of genetic diversity. In this study, the PIC for each locus was vary from 0.076 – 0.673 (Table 3). Normally, a high reliability marker for the pedigree analysis is shown by PIC value greater than 0.500 [26]. There are four markers showed PIC value more than 0.5, namely TAA15, TAA41, CAT01, and AG14.

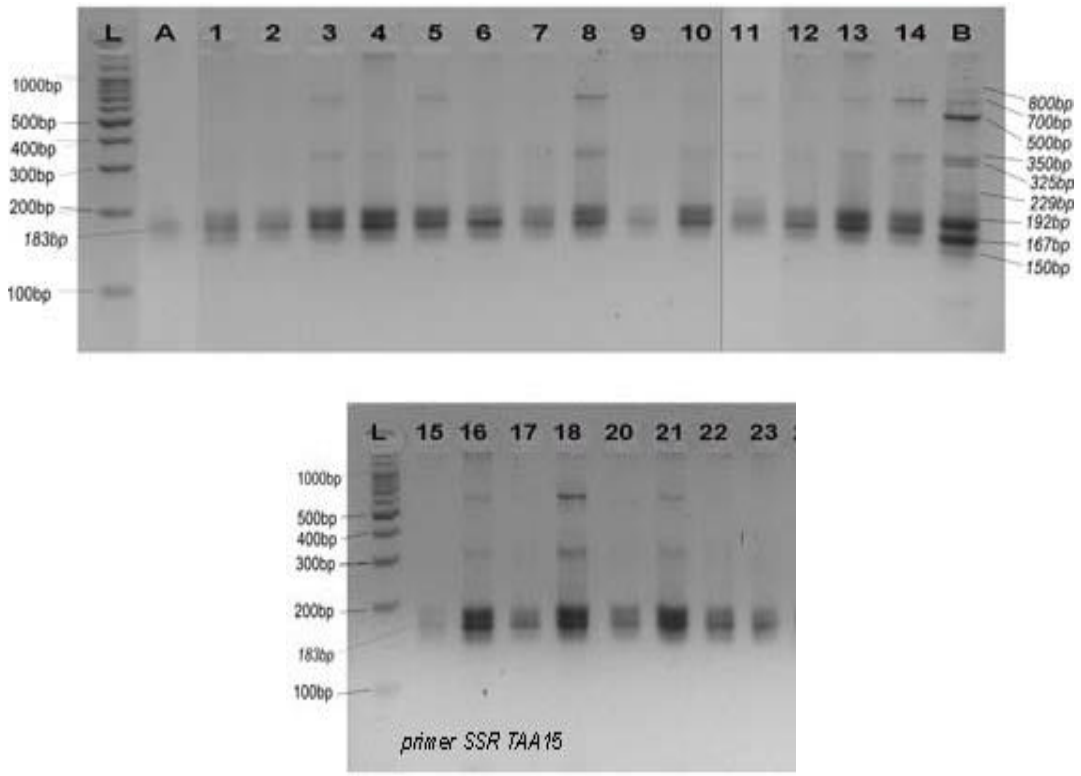

Fig 1. The SSR profiles of 23 somatic hybrid genotypes showing allelic variation amplified by TAA15 marker.Lane L, molecular mass marker (100 bp DNA ladder), Lane A,B, parental (Siam Madu and Satsuma Mandarin) .

All SSR alleles from 5 markers scored were used for genetic diversity analysis. Dice's similarity coefficients were calculated to assess the genetic resemblances among the genotypes and the similarity coefficients matrix was used for UPGMA cluster analysis (Fig 2). The dendrogram based on genetic similarities showed that the 23 genotypes and their parents formed four major clusters. Cluster I, consists of F3, F7, F10, F14 F18, F22 and Siam Madu with 89% genetic similarity. Cluster II consist of F1, F5, F8, F6, F9, F11, F12, F13, F15, F16, F19, F20, F21 and F23 with 91% genetic similarity. Cluster III consist of F17 and cluster IV consist of Satsuma Mandarin, with 58,6% and 24% genetic similarity, respectively. The genetic distance between all somatic hybrid genotypes with the parental Satsuma Mandarin is 0.60, except for F17 the genetic similarity is 0.34.

Our study could identify 23 somatic hybrid genotypes from Siam Madu and Satsuma Mandarin using 5 SSR markers. This result confirmed the result of other studies that used several molecular technique separately, such as Barkley *et al*. [18] and Golein *et al*. [27] clarified that SSR markers was valuable tool for cultivar identification, germplasm diversity and phylogenic studying for differentiation among close genotypes of Citrus. SSR markers are also effectively used for identification of any kind hybrids in citrus. Oliveira *et al.* 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 [28]. Even Fu *et al.* performed SSR analysis of somatic hybrids between *Citrus sinensis* (navel orange) and *Clausena lansium* (sweet wampee) [29]. SSR analysis of seven randomly selected tetraploids and three triploids showed that they had specific fragments from both fusion parents, thereby confirming they are hybrids. Ferrante *et al.* determined the allelic configuration of eight new citrus tetraploid hybrids using SSR markers to observe capillary electrophoresis and PCR based dosage effects [30].. 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).

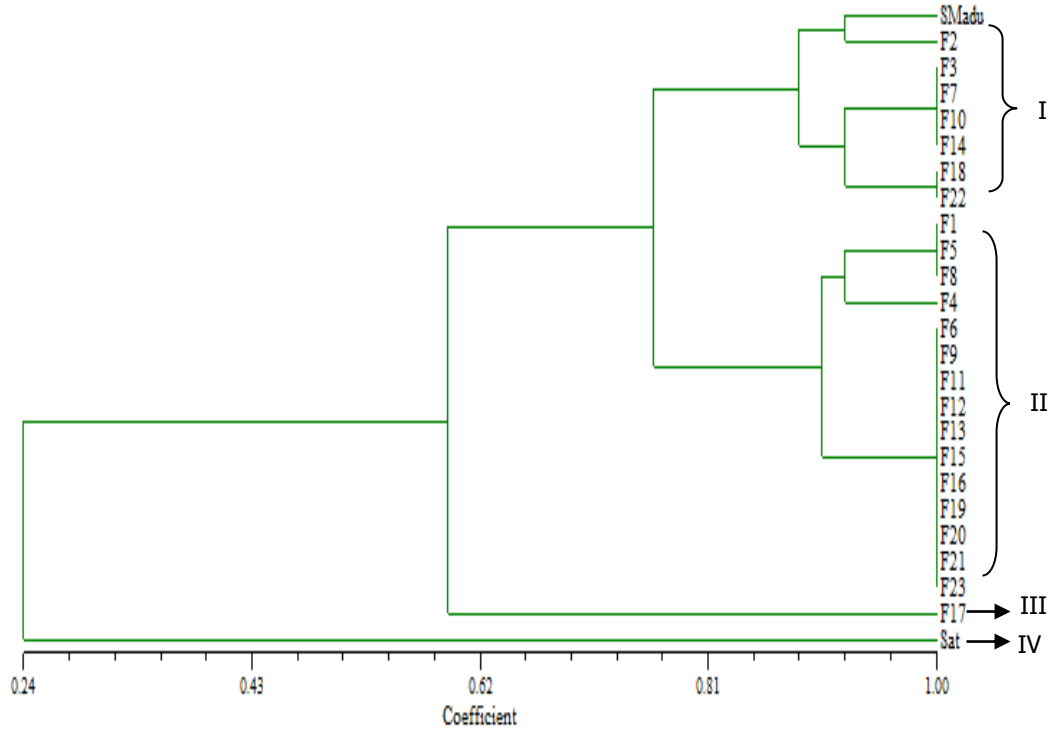

Fig.2. Dendrogram showing similarity coefficients and genetic relationships among 23 somatic hybrid genotypes between Siam Madu and Satsuma Mandarin and their parental analyzed by SSR.

CONCLUSION

In this research, 23 citrus genotypes from protoplat fusion between "Siam Madu" and "Satsuma Mandrin", already observed by five SSR primer markers. All genotypes were classified into 4 clusters based on their genetic similarity with parents. Cluster I consists of 6 genotypes, Cluster II consist of 14 genotypes , Cluster III consist only one genotype, and Cluster IV consist of Satsuma Mandarin.

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