Indian Journal of Agricultural Sciences 90 (12): 2307–12, December 2020/Article
https://doi.org/10.56093/ijas.v90i12.110313

Microsatellites assisted rapid identification of mandarin hybrids and assessment of their phenotypic variability

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Received: 27 December 2018; Accepted: 18 May 2020

ABSTRACT

Kinnow (*Citrus nobilis* × *C. deliciosa*) is a commercially important mandarin but contains large number of seeds. Mukaku Kishu (*C. kinokuni*), a seedless mandarin can be used to transfer seedlessness in Kinnow through hybridization. But, the polyembryony in Kinnow and absence of any heritable morphological trait in Mukaku Kishu are limiting factors in early identification of hybrids. SSR markers due to their high polymorphism and co-dominant nature are useful for hybrid identification. We performed hybridization between Kinnow (♀) and Mukaku Kishu (♂) and differentiated hybrids using SSR markers. A total of 134 SSR markers were tested for polymorphism between the parents; 20 were found polymorphic while four (CMS04, Ci06A05b, CiBE1500 and TAA15) were suitable for hybrid detection. The hybrid detection ability varied among the markers. The marker CMS04 identified 67 hybrids while the remaining three markers independently identified 104 hybrids from the total analysed 697 seedlings. The hybrid seedlings differed for height and number of spines per plant. The two traits also showed moderate positive association (Pearson correlation = 0.69). The study demonstrated the practical utility of SSR markers in enhancing the efficiency of breeding programmes in absence of distinct morphological distinguishers for hybrid detection. The identified hybrids progeny will serve as a foundation for identification of potentially seedless candidates.

Key words: Hybrid detection, Mandarin, Microsatellite markers, Seedlessness, Trait variability

Citrus fruits are most economically important fruit crops. In India, citrus ranks third in production after banana and mango. It is cultivated over an area of 1.0 million ha with a production of 12.5 million metric tonnes (Anonymous 2018). Mandarin (35.5%), followed by sweet oranges (29.9%) and limes and lemons (21.05%) are the major citrus types being cultivated in the country. Among the mandarins, Kinnow (*C. nobilis* × *C. deliciosa*) is a commercially important variety of north-western India. It is high yielding and fruits have abundant juice with good flavor. However, Kinnow fruits contain large number of seeds. The high seed content not only cause inconvenience in its fresh consumption but also reduces its utility into juice industry. Of the different fruit parts, seeds also cause accumulation of maximum limonin (2700 ppm), a bitter principal responsible for inducing delayed bitterness in Kinnow juice (Arora *et al.* 2018). Genetic improvement of Kinnow for seedlessness would inevitably enhance its edible value, processing value and export prospects.

The Mukaku Kishu or seedless Kishu (*C. kinokuni*) is a potential source of seedlessness (Yamasaki *et al.* 2007). This is a bud mutant of the original seedy variety Kishu Mikan. The fruits of this mandarin are small in size, delicious, easy to peel, tender, juicy, sweet and fragrant. Mukaku Kishu has diploid chromosome number, i.e. 2n=18 and produces completely seedless fruits even after cross pollination (Befu *et al.* 2002). Its seedlessness is caused by an arrest of seed development at an early stage (Yamasaki *et al.* 2009). Seedlessness of Mukaku Kishu was found to inherit in 1:1 ratio in the progeny when crossed with seedy cultivars (Nesumi 2001). Nesumi (2001) proposed that seedlessness in *C. kinokuni* was regulated by genes, *i.e. Fs* dominant gene and *Is* repressor gene (inhibits seedlessness), with the Mukaku Kishu genotype being *Fsfs-isis*. Attributing to single dominant gene regulation, seedlessness from Mukaku Kishu can be introgressed in Kinnow by using Mukaku Kishu as the pollen donor. But, Kinnow like many other citrus produces polyembryonic seeds. In polyembryony, multiple embryos of nucellar origin (genetically identical to the mother parent) grow together with the zygotic embryo in the same seed, which not only restrict the development

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of zygotic embryos (Soares Filho *et al.* 1992), but also create problems in correct identification of hybrids post germination. Due to long juvenile phase, large tree size of *Citrus* and prevalence of polyembryony, it is important to identify the hybrids at seedling stage. However, Mukaku Kishu does not exhibit any unique morphological feature of dominant inheritance that can be employed to differentiate hybrids from the Kinnow type nucellar seedlings at seedling stage. In absence of morphological differences, molecular markers are useful to elucidate the genetic differences between the individuals. Different DNA markers like restriction fragment length polymorphism (RFLP) (Carimi *et al.* 1998), randomly amplified polymorphic DNA (RAPD) (Bastianel *et al.* 1998), simple sequence repeats (SSRs) (Ruiz *et al.* 2000, De Oliveira *et al.* 2002) and single nucleotide polymorphism (SNPs) (Zhu *et al.* 2013) have been used for selecting the hybrids from a mixed stand of nucellar and zygotic seedlings in *Citrus*. However, SSRs due to their co-dominant inheritance, high variability, ease of use and detection, and reproducibility, are the favourite marker for hybrid detection in citrus breeding (Hazarika *et al.* 2014). The study aimed to develop Kinnow × Mukaku Kishu hybrids, their differentiation from nucellar seedlings using polymorphic SSR markers and recording of phenotypic variability in the progeny at seedling stage.

MATERIALS AND METHODS

The flowers on Kinnow trees were emasculated and cross pollinated with Mukaku Kishu pollen at Punjab Agricultural University, Dr J C Bakhshi Regional Research Station, Abohar during the year 2016. The seeds from the crossed fruits were extracted at full maturity and were sown in seedling trays prefilled with sterile potting mixture. The seedlings derived from every seed were then transplanted in polybags during March 2017. The seedlings were thereafter kept in a green house.

DNA was extracted from young leaves of 4-5 months old seedlings using modified CTAB method as described by Naliath *et al.* (2017). The DNA quantification was performed using Bio-spectrometer (Eppendorf, Hamburg, Germany) and quality was checked through electrophoresis on 0.8% agarose gel. The concentration of DNA was adjusted to 25 ng/µl for use in polymerase chain reactions.

In order to identify the polymorphic SSR markers, the Kinnow and Mukaku Kishu genomes were tested with 134 SSRs. The SSRs represented different repeat motifs: 72 with di-nucleotide motif, 38 with tri-nucleotide motif, 12 with tetra-nucleotide, 5 with penta-nucleotide and 7 were of compound nature. For SSR amplification, the PCR was performed in 10 µl volume containing 50 ng genomic DNA, 1X Go Taq Flexi PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of primers (forward and reverse each) and 0.75 units of Taq polymerase. The final volume was made by adding sterile double distilled water. The amplification was performed in an Eppendorf thermo-cycler (Eppendorf, Hamburg, Germany) programmed as: initial denaturation at 94ºC for 5 min, followed by 30-35 cycles of 30 seconds at 94ºC, 40 seconds at 40-52º C (depending upon the annealing temperature of the primer), 1.5 minutes at 72º C, with a final extension of 72ºC for 10 minutes. The amplified products were resolved on 2.5 % agarose gel containing ethidium bromide and the products were visualized under photo gel documentation system (UVP, Upland, CA, USA). The hybrids were identified by comparing the banding pattern of the individuals with the parents.

The identified hybrids were characterized for leaf shape, plant height and number of spines per plant. The leaf shape in the parents and progeny individuals was assigned as per the International Plant Genetic Resources Institute (IPGRI) - Citrus descriptor. To compare the growth of the parents, the plant height and canopy volume were measured in mature (10 year old) and young (1 year old) plants of Kinnow and Mukaku Kishu. The plants were raised on the rough lemon rootstock. The distribution of plant height and number of spines in the progeny was checked through the 'Box and Whisker' plot. The association of plant height with spine number in the hybrids was also examined through correlation analysis.

RESULTS AND DISCUSSION

Parental polymorphism assay

Of the 134 tested SSRs, 20 (16.7%) showed polymorphism between the parents- Kinnow and Mukaku Kishu. In terms of number of repeat motifs, 12 di-nucleotide (16.7%), 5 tri-nucleotide (13.2%), 1 tetra-nucleotide (8.3%) markers exhibited polymorphism (Fig 1**).** The remaining two polymorphic SSRs were of compound type (Fig 1).

Table 1 Summary of SSR markers used for hybrid detection and their hybrid detecting ability

Marker name	Sequence (5'-3')	Annealing temperature (°C)	Hybrids detected[a]
TAA15 F	GAAAGGGTTACTTGACCAGGC	52	104
TAA15 R	CTTCCCAGCTGCACAAGC		
CMS04 F	CCTCAAACCTTCTTCCAATCC	47	67
CSM04 R	CTGTAAAGTACATGCATGTTGG		
Ci06A05b F	TCTCTGGTTGGTTTTTGTGA	46	104
Ci06A05b R	ATGATGAAAAGCAAGGGG		
CiBE1500 F	AAATAACGACGAAACACTTGA	45	104
CiBE1500 R	GCAGTAAATGGAGGAGAATAA		

A total of 697 seedlings derved from 687 seeds were used or SSR analysis.

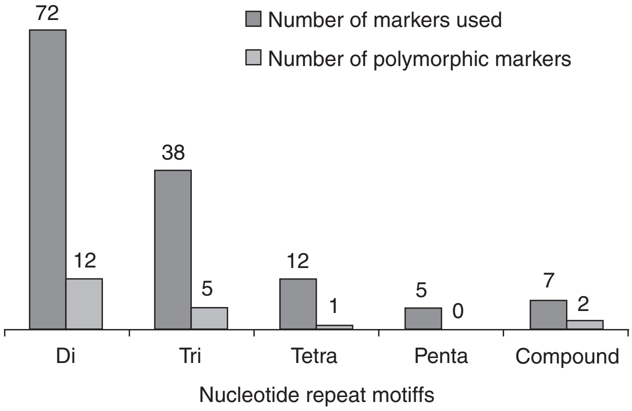

Fig 1 Influence of nucleotide repeat motifs of SSRs on their polymorphism.

The decrease of polymorphism with increase in the repeat units is consistent with the findings of Biswas *et al.* (2014) for genome wide SSRs characterization in sweet orange. The polymorphism at a SSR locus results from the variation in the number of repeat units. The shorter repeat motifs (mono or di-nucleotides) can accommodate more stretch of repeats than longer motifs such as tetra-, penta-, and hexa-nucleotides (Xu *et al.* 2013), thus exhibit higher polymorphism. Unexpectedly, the compound markers revealed the highest polymorphism (28.03%), which possibly could be due to testing of less number of markers under this category. Out of the polymorphic SSRs, four SSRs namely CMS04, Ci06A05b, CiBE1500 and TAA15 were finally selected to identify the true F_1 hybrids (Table 1).

Hybrid identification

A total of 697 seedlings originating from 687 seeds were characterized with four SSR markers. A total of 104 (15.1%) seedlings were found to be hybrids. However, the hybrid detection efficiency varied among the four SSR markers. The SSR marker CMS04 could unambiguously detect only 67 hybrids, whereas the rest 3 markers (TAA15, Ci06C05b and CiBE1500) independently identified 104 hybrids. In our study, the differential ability of the markers to sort hybrids was possibly related to their allelic states in the parents. The CMS04 amplified two different bands for Mukaku Kishu, both of which were polymorphic with the single band in Kinnow. However, many of the individuals confirmed as nucellars by other three markers amplified a stutter band of size similar to upper band of Mukaku Kishu (Fig 2a), which created problem in clear demarcation of hybrids. For this marker, the individuals amplifying lower Mukaku Kishu band could only clearly be taken as hybrids (Fig 2a). Stuttering is one of the recognized sources of causing error in microsatellite genotyping (Dewoody *et al.* 2006). The rest three markers TAA15, Ci06C05b and CiBE1500 amplified stutter free homozygous alleles in the parents and were able to identify the 104 hybrids independent of each other (Fig 2b & c).

In past, the SSRs have been deployed for hybrid identification either in complementation with some morphological index (De Oliveira *et al.* 2002, Yun *et al.* 2007) or solo (Ruiz *et al.* 2000, Rao *et al.* 2008) in citrus. The present study demonstrated the practical utility of SSR markers in early differentiation of hybrids from the nucellars in absence of strong morphological clue. The results showed that the markers displaying explicit polymorphism may be more useful for this purpose. Early sorting of hybrids would not only reduce the cost incurred on the growth and maintenance of nucellar seedlings, but will also allow efficient utilization of the space and resources in the breeding programme.

Phenotypic variability in the progeny

The parents (Kinnow and Mukaku Kishu) and hybrid seedlings were characterized for leaf shape, plant height (cm) and number of spines per plant. Both the parents had elliptic leaves.The shape of leaves in hybrids was either elliptic or intermediate between elliptic to lanceolate, without any clear cut differentiation. Leaf is one of the key traits that assist in taxonomic classification of citrus. The true citrus fruits fall under tribe *Citreae* and subtribe *Citrenae* of the subfamily *Aurantioideae* (Swingle and Reece 1967). Within the subtribe, the genera *Poncirus* and *Citropsis* bear trifoliate and multifoliate leaves, and show dominant inheritance pattern over unifoliate leaves present in *Citrus* (Tan *et al.* 2007, Smith *et al.* 2013). However, the differences among the individuals bearing unifoliate leaves are difficult to quantify (Iwata *et al.* 2002).

The variation in plant height and number of spines per plant was analysed through ‘Box and Whisker Plot’. The plant height in the hybrid individuals ranged from 7.2 to 58.0 cm (Fig 3a), representing an 8 fold variation for this trait. In

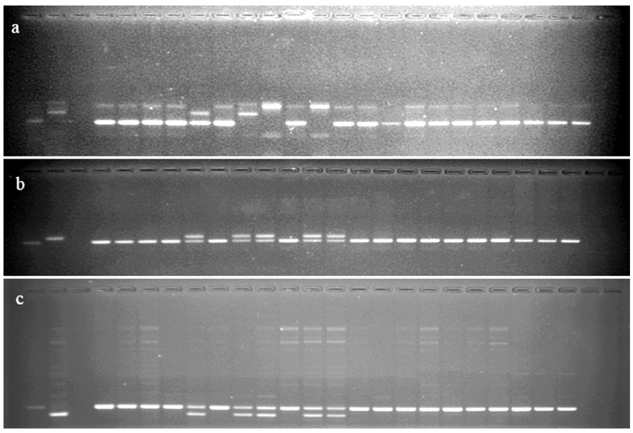

Fig 2 Differentiation of Kinnow’ × ‘Mukaku Kishu’ hybrids from ‘Kinnow’ derived nucellar seedlings through SSR markers CMS04 (panel a) Ci06A05b (panel b) and CiBE1500 (panel c). In the panels, lanes 1: ‘Kinnow’, 2: ‘Mukaku Kishu’, 3: negative control and 4 to 24 are seedlings used for hybrid detection. The individuals at 8, 10, 11 and 13 were identified as hybrids by all the markers whereas the individual 14 was confirmed as hybrid only by markers Ci06A05b and CiBE1500. The faint top band in CMS04 was a stutter and created ambiguity in scoring.

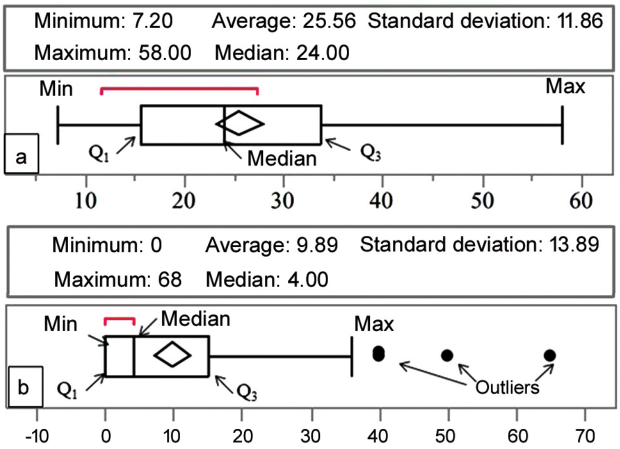

Fig 3 Measurement of variability of plant height (a) and number of spines per plant (b) in Kinnow × Mukaku Kishu hybrid progeny through 'Box and Whisker' plot.

box plot, the plant height for the first 75% individuals was distributed from 7.2 to 33.0 cm, while the remaining 25% hybrids displayed variation of higher magnitude that ranged from 33.0 to 58.0 cm (Fig 4a). The spines were present in the range of 0 to 35 per plant, and four hybrids behaved as the outliers (Fig 3b and 4b). The hybrids in the lower half of the box plot had spines ≤ 4, while 25% of which were spineless. The hybrids in the upper half of the box plot displayed a great amount of variation for the number of spines, varying from 4 to 35 (Fig 3b). The mature (10 year old) trees of Kinnow and Mukaku Kishu differed in plant height (3.3 m versus 2.7 m) and canopy volume (66.9 m^3 versus 52.6 m^3). Similarly, when compared between 1 year old nursery plants of Kinnow and Mukaku Kishu, these also showed differences in plant height (0.74 m versus 0.62 m) and canopy volume (0.09 m^3 versus 0.03 m^3).

The genetics of plant height has been reported for *Poncirus trifoliata* cv. Flying Dragon where in 'dwarfness' is controlled by a single dominant gene (Cheng and Roose 1995). The condition of thorniness is also reported to be variable among different *Citrus* types, indicating it is also controlled genetically in this genera (Spiegel-Roy and Teich 1972). Spiegel-Roy and Teich (1972) concluded that the two histogenic layers L_1 and L_2 determine the thorny phenotype, which in turn may be regulated by few dominant genes. The exact inference about the inheritance of the traits: plant height and spines in the Kinnow × Mukaku Kishu progeny was limited by the unavailability of seed derived Mukaku Kishu plants as it is seedless. However, Kinnow produced spines in nucellar seedlings as well as on the adult trees budded on rough lemon rootstock. In contrast, budded mature Mukaku Kishu trees were spineless (Fig 4b). Similarly, the mature trees also displayed growth differences. Therefore, the variation for the two traits in the hybrids progeny is due to the genetic contribution of two parents.

The association between plant height and number of spines per plant in the hybrid progeny was checked and these recorded moderate positive association (Pearson correlation= 0.69) (Fig 5). In this association, majority of the short statured hybrids were either spineless or had low spine number and taller hybrids had in general higher number of spines per plant. In contrast to our findings, a negative relationship was shown between plant height and spines by Cheng and Roose (1995) for *Poncirus*. The contrasting results could be due to the differences of genera.

In conclusion, the study demonstrated the utility of SSR markers in hybrids identification in absence of any morphological clue, thus increasing the effectiveness of the breeding program. The SSR markers Ci06A05b and CiBE1500 are now being regularly used to identify Kinnow × Mukaku Kishu hybrids. The variation within the progeny individuals for plant height and spines reflects the segregation in the progeny and embarks the contribution of the two parents. The progeny expectedly will provide a base for selection of superior seedless mandarin hybrids.

Fig 4 Spectrum of variation in the progeny individuals for plant height (a) and spine differences in twigs of mature parents and the progeny individuals (b).

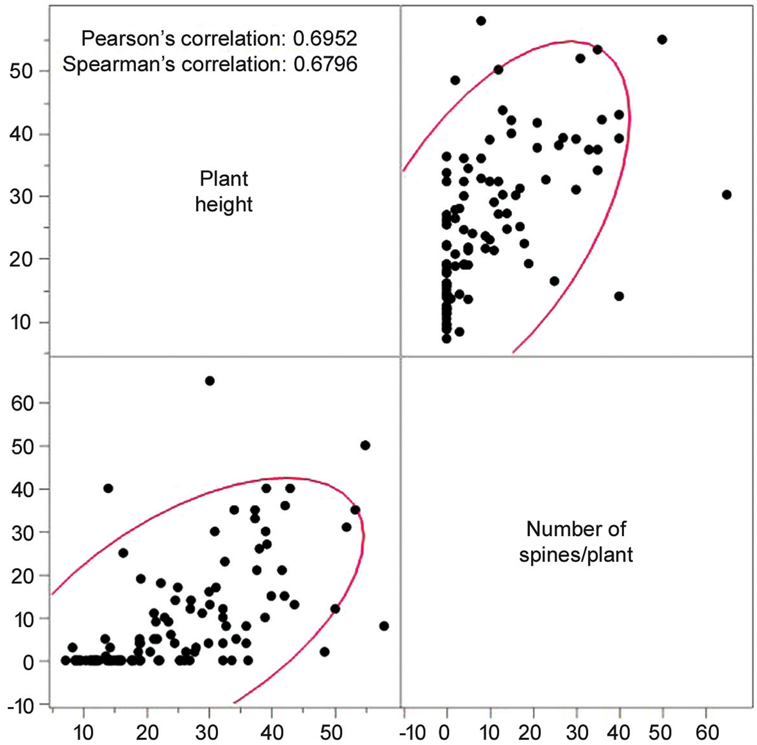

Fig 5 Association of plant height and number of spines per plant in the Kinnow × Mukaku Kishu progeny.

ACKNOWLEDGEMENT

The grant received from the Indian Council of Agricultural Research, New Delhi under the NAE project- 'Genetic improvement of Kinnow mandarin for fruit quality, biotic and abiotic stress tolerance' [F. No. 10(2)/ 2014- EP & HS] to conduct this research is gratefully acknowledged.

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