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IDENTIFICATION OF CITRUS POLYPLOIDS USING CHROMOSOME COUNTS, MORPHOLOGICAL AND SSR MARKERS

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Polyploids play a key role in the improvement of citrus cultivars. Citrus breeding programs necessitate morphological, cytological and genetic characterization of cultivars. Here we analyze the polyploids using these three approaches. Cytological studies signify occurrence of spontaneous triploids (9.95%) and tetraploids (6.05%) in Kinnow mandarin and Succari sweet orange, respectively. Leaf characteristics were found directly proportional to the ploidy level; they change with an increase in the ploidy level. Genetic variability amongst morphologically distinct polyploids was confirmed using Simple Sequence Repeat (SSR) molecular markers. Accessions K_1, K_4, K_5 and K_7 of Kinnow, F_7, F_8 and F_{10} of Feutrell's Early, M_2 and M_4 of Mosambi, and S_1 and S_{11} of Succari were found polymorphic, and are considered useful in current breeding programs in Pakistan. SSR loci genotyping, morphological selection and cytological screening of polyploids appeared to be an improved strategy for efficient screening and characterization of polyploids at seedling stage.
Keywords: Genetics, phenotype, aborted seeds, ploidy, Kinnow

INTRODUCTION

The genus *Citrus*, member of family Rutaceae, includes a wide range of species, varieties and cultivars. Taxonomic relationships within *Citrus* are complex due to frequent occurrence of fertile interspecific hybrids and spontaneous mutants in nature; however, recent developments in molecular biology revealed only three true primary species namely, mandarins, pummelos (*C. maxima* L.) and citron, *C. medica* L. (Moore, 2001). Rest of the species are secondary and arose from interspecific hybridization of the primary species followed by clonal propagation (Ollitrault *et al.*, 2012). *Citrus* industry is mainly confronted by problems like lack of genetic diversity, low productive age and yield, high number of seeds, pests and diseases and huge post-harvest losses. Polyploids have played a significant role in evolving and exploring genetic diversity in crop plants particularly *Citrus*. Frequency of polyploids in flowering plants range from 47 to 70% including the world's most important horticultural crops such as *Citrus*, banana, apple, grapes, strawberry and potatoes (Hilu, 1993). Polyploids are a success story because of having higher heterozygosity than the diploid progenitors, less inbreeding depression, genome rearrangements, higher probability of divergent evolution in duplicated genomes, seedlessness, gigantism and higher genetic diversity. Furthermore, genome doubling has potential to bring populations more adapted to adverse climates.

Polyploids can be obtained by exploring spontaneous doubling events in nature such as triploids, tetraploids, pentaploids, hexaploids and octaploids available in *Citrus* and can also be created artificially by interploid and interspecific hybridisation, somatic doubling by colchicine and through endosperm culture *in vitro* (Usman *et al.*, 2002, 2008). Although recovery of spontaneous polyploids compared to interploid and somatic hybridisation significantly reduces the time required for polyploid development from 5-10 years up to 1-2 years, it has to be followed by intensive cytological and morphological screening for the confirmation of putative polyploids. Even then correct identification of hybrids is hindered due to the complex reproductive behavior of the plant including polyembryony, long reproduction cycle, sterility (juvenility), incompatibility and endogamy (selfing) depression (Grosser *et al.*, 1996).

Different procedures have been developed for germplasm screening at early stages of plant development including colorimetric, spectroscopic, morphological, chromatographic and isoenzymatic (Anderson *et al.*, 1991); however, none of these methods offers absolute certainty about the nature of selected seedlings. Exploring DNA polymorphisms with genetic markers for linkage mapping (Sahin-Cevik and Moore, 2007) and for the identification of putative polyploids can speed up screening of the progenies. The discovery of microsatellite sequences or Simple Sequence Repeats (SSR) by Tautz *et al.* (1986) significantly improved the detection of polymorphism in many plants. The SSR

markers have many advantages being: 1) co-dominant and PCR based, 2) multi-allelic and hyper-variable, 3) randomly and uniformly distributed throughout the eukaryotic genome, and 4) accessible via published primer sequences (Saghai-Maroof *et al.*, 1994). Hence, SSR markers are very useful for genetic characterization and linkage mapping (Breto *et al.*, 2001; Pang *et al.*, 2003; Riahi *et al.*, 2011). In addition, polymorphisms of SSRs can be detected by PCR and an automated DNA sequencer at the seedling stage for selection in practical plant breeding and biotechnology programs. Highly polymorphic SSR loci are also valuable tools for identification of *Citrus* lines/cultivars and for genetic diversity analysis among the germplasm. This article describes successful early detection of polymorphisms in spontaneous polyploids through a combined application of cytological and morphological screening followed by SSR analysis of the morphologically distinct polyploids.

MATERIALS AND METHODS

***Plant materials*:** Putative spontaneous polyploids from 500 underdeveloped and 300 shrivelled seeds of each *Citrus* cultivars; Kinnow, Feutrell's Early mandarins (*Citrus reticulata* Blanco) and Mosambi, Succari sweet oranges (*Citrus sinensis* Osbeck) were raised on MT (Murashige and Tucker, 1969) medium under *in vitro* conditions. The fruit material used was collected from Experimental Fruit Garden, Institute of Horticultural Sciences, University of Agriculture, Faisalabad-Pakistan following Fatima *et al.* (2010).

Chromosome counting and morphological screening: Chromosome counts were made from root-tip sections collected from plant populations (Figure 1a) at the time of transplant to pots from 8:30 a.m. to 11:00 a.m. After roots were pre-treated, samples were prepared and examined under Nikon Optiphot Fluorescence Microscope at ×1250. One-year-old potted polyploids were maintained in the greenhouse conditions for their morphological characterization (IPGRI, 1999).

***Stomatal studies*:** Polyploids were grouped into diploids, triploids and tetraploids based on chromosome counts. These ploids were further evaluated for the confirmation of ploidy through stomatal studies comprising analysis of number of stomata, size of stomata (i.e., length and width) following Khan *et al.* (1992) and chloroplast density in the guard cells. Fresh samples were taken early morning from 8.30 to 11.00 a.m. so that fully open and turgid guard cells may be achieved. The lower epidermis was removed by piercing leaf with hand and placed on the glass slide after addition of one drop of distilled water. The size of stomata and chloroplast density was scored under the microscope at x125 magnification. Five stomata were observed per leaf. The experiments were laid out according to Completely Randomized Design (CRD) with three repeats and factorial arrangement. Data were analysed and means were compared by New Duncan's Multiple Range (DMR) Test (Damon and Harvey, 1987).

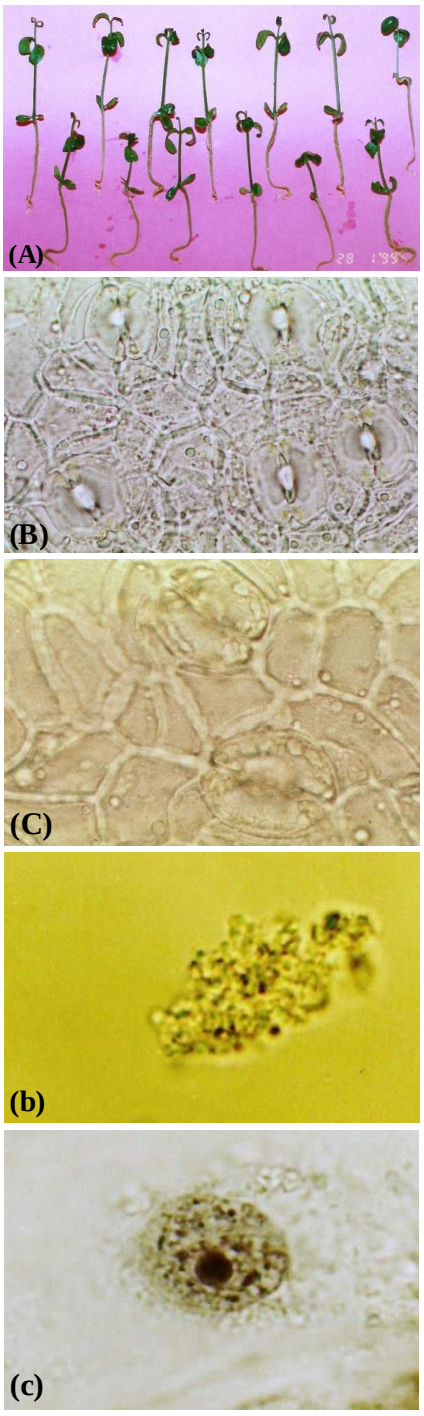

Figure 1. ***In vitro*** **raised Kinnow mandarin seedlings (a), stomatal density (125×) and chromosomal counts (1250×) in putatitive polyploids B, b) diploids and C, c) polyploids.**

SSR analysis: Total cellular DNA of Citrus polyploid leaves was extracted with little modifications as described by Spychalla and Bevan (1993). The extracted DNA (1-2µl) was used as a template for PCR following protocols (Saiki et al., 1985). For SSR analysis controls included diploid cultivars of Kinnow, Feutrell's Early, Mosambi and Succari. Primer sets were designed to amplify 100-250 base pairs fragment of Citrus nuclear DNA for locus I (a) and locus II (b) using Primer 3 software. Several PCR primer sets were used in SSR analysis using Master Cycler Gradient PCR machine (Eppendorf®, Germany), however following sequences successfully detected polymorphism in the used germplasm at annealing temperature 54°C.

Forward (F1) 5'-TTCTCCTCATCTTCGACACG-3'
Reverse (R1) 5'-CCGATCTCTTACGACGTAACAT-3'
Forward (F2) 5'-ATCTGTGTGAGGACAGAT-3'
Reverse (R2) 5'-CCTCTATTAATGTGCGTC-3'.

PCR products were gel electrophorised on 0.8% to 1.5% (w/v) agarose gels, containing 0.5× TAE buffer and 0.5 µg/mL ethidium bromide depending on the size of the DNA fragments to be separated and bands were visualized under UV light.

RESULTS

***Morphological and cytological screening*:** Chromosome counting revealed the highest occurrence of spontaneous triploids (9.95%) in Kinnow mandarin followed by Mosambi sweet orange and tetraploids in Kinnow mandarin and Succari sweet orange (Fig. 1b, c; Fig. 2). Mandarin cultivar; Feutrell's Early showed the lowest percentage recovery of triploids while Feutrell's Early and Mosambi cultivars were the lowest in recovery of tetraploid plants.

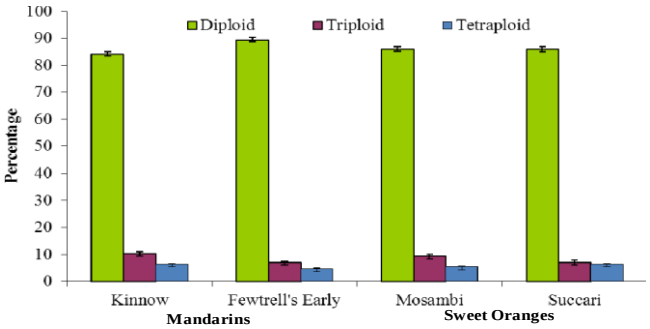

Figure 2. Frequency of polyploids recovered from open pollinated seeds in *Citrus* cultivars.

Table 1. Morphological comparison of diploid, triploid and tetraploid plants of *Citrus*.

Cultivars	Morphological Characters	Ploidy Level		
		2n	3n	4n
Kinnow (Mandarin)				
	Angle of leaf petiole (degree)	48.08 b	48.71 ab	49.14 a
	Leaf lamina length (cm)	6.46 c	8.72 b	9.34 a
	Leaf lamina width (cm)	3.88 c	4.68 b	5.29 a
	Ratio leaf lamina (length/width)	1.66 b	1.80 a	1.58 c
	Length of petiole (cm)	1.42 a	1.24 b	1.29 b
	Stem girth (cm)	0.71b	0.73 b	0.79 a
Feutrell's Early (Mandarin)				
	Angle of leaf petiole (degree)	44.42 b	45.31 b	48.70 a
	Leaf lamina length (cm)	6.29 bc	6.55 b	7.23 a
	Leaf lamina width (cm)	4.92 a	3.34 b	3.66 b
	Ratio leaf lamina (length/width)	1.27 c	1.55 b	1.94 a
	Length of petiole (cm)	1.68 a	1.27 b	1.52 ab
	Stem girth (cm)	0.37 c	0.49 b	0.59 a
Mosambi (Sweet orange)				
	Angle of leaf petiole (degree)	48.34 b	45.56 c	50.24 a
	Leaf lamina length (cm)	6.44 ab	6.59 a	6.57 a
	Leaf lamina width (cm)	3.63 ab	3.94 a	3.92 a
	Ratio leaf lamina (length/width)	1.77 a	1.66 ab	1.66 ab
	Length of petiole (cm)	1.58 bc	1.85 a	1.67 b
	Stem girth (cm)	1.12 ab	1.16 a	1.16 a
Succari (Sweet orange)				
	Angle of leaf petiole (degree)	42.55 b	41.97 b	45.29 a
	Leaf lamina length (cm)	4.82 c	6.35 b	7.89 a
	Leaf lamina width (cm)	2.44 c	3.55 b	4.04 a
	Ratio leaf lamina (length/width)	1.97 a	1.74 b	1.79 ab
	Length of petiole (cm)	1.38 c	1.65 a	1.57 b
	Stem girth (cm)	0.51 b	0.56 ab	0.58 a

*Means sharing the same letters within cultivar are statistically non-significant

Table 2. Stomatal studies in citrus polyploids.

Cultivars	Stomatal Characters	Ploidy Level		
		2n	3n	4n
Kinnow (Mandarin)				
	No. of stomata (per cm^2)	7.3 a	5.8 b	4.7 c
	Width of stomata (µ)	16.3 c	18.9 b	22.0 a
	Length of stomata (µ)	20.3 c	22.1 b	26.3 a
	No. of chloroplasts	8.3 b	9.8 b	13.4 a
Feutrell's Early (Mandarin)				
	No. of stomata (per cm^2)	7.7 a	6.3 b	5.7 c
	Width of stomata (µ)	16.7 c	17.7 b	18.7 a
	Length of stomata (µ)	20.3 c	22.0 b	23.7 a
	No. of chloroplasts	7.8 c	10.4 b	12.2 a
Mosambi (Sweet orange)				
	No. of stomata (per cm^2)	8.7 a	7.7 b	6.3 c
	Width of stomata (µ)	18.2 c	19.7 b	20.3 a
	Length of stomata (µ)	23.3 b	24.0 a	22.7 c
	No. of chloroplasts	7.0 b	8.7 a	8.3 a
Succari (Sweet orange)				
	No. of stomata (per cm^2)	8.3 a	7.3 b	6.7 c
	Width of stomata (µ)	17.7 c	18.0 b	19.5 a
	Length of stomata (µ)	20.3 c	21.7 b	23.3 a
	No. of chloroplasts	6.3 c	9.3 b	10.7 a

***Means sharing the same letters are statistically non-significant**

Foliage studies of the putative ploids were found directly proportional to the ploidy level as most of the morphological parameters studied increased with increase in the ploidy. Tetraploid seedlings of both mandarins and sweet oranges were found significantly higher for angle of leaf petiole, length and width of leaf lamina and stem girth compared with diploid and triploid plants. Diploids were high in width of leaf lamina and length of petiole. However, triploids showed intermediate values for all the parameters studied (Table 1). Leaf shape was elliptic to lanceolate in different cultivars.

Stomatal studies of spontaneous ploids revealed that number of stomata significantly decreased (8.7 to 4.7) with increase in the ploidy level (2n to 4n) in all the citrus cultivars including Kinnow, Feutrell's Early, Mosambi and Succari. The length (16µ-22µ) and width (20µ-26µ) of stomata and number of chloroplasts (6.3-13.4) increased with increase in the ploidy level from diploid to tetraploids (Table 2). The above findings indicate that cytological and morphological measures of screening are helpful in identification of polyploids and or mixoploids out of large plant populations which would have been difficult to screen otherwise. This pre-screening facilitates SSR locus based genotyping by reducing the number of samples to be analyzed.

***Molecular marker-based genotyping of Citrus polyploids*:** In mandarins, SSR analysis was carried out in morphologically distinct polyploids. The diploid control was found homoallelic for locus I in Kinnow and for both loci in Feutrell's Early whereas it was heteroallelic for locus II in Kinnow and size of the three alleles ranged from 100-250 bp. K_1, K_4, K_5 and K_7 polyploids were found heteroallelic while bands having double and triple dose alleles appeared brighter, such as K_1 for locus II, K_5 for locus I, K_2 and K_9 for both loci confirming their polyploid nature, compared with faint bands which appeared as single dose allele (Fig. 3a,b). Moreover a unique band (260-300 bp) was marked at locus II in K_1 (arrow) which could be helpful in distinguishing K_1 from rest of the genotypes. In case of Feutrell's Early cultivar F_7-F_9 were found heteroallelic for locus I and F_6-F_7 for locus II, while F_5, F_8 and F_{10} were homoallelic for locus II. Rest of the polyploids depicted brighter bands due to higher allelic dosage (Fig. 3c-d). Interestingly, common band (upper) for locus I was present in all the genotypes (F_o-F_9) and absent in F_{10}, however, the amplified band in F_{10} (lower) was common amongst F_7-F_{10} genotypes suggesting that F_7-F_9 are heteroallelic having both the alleles of F_o-F_6 genotypes (upper) and F_{10} genotype (lower; Figure 4c). Accessions F_7 and F_{10} also showed unique bands (lower) at locus I which were absent in rest of the genotypes thus distinguishing F_7 and F_{10} for locus I with rest of the genotypes.

In sweet oranges, diploid control was heteroallelic for both loci in Mosambi (Fig. 4a-b) and for locus II in Succari whereas it appeared homoallelic for locus I in Succari (Fig. 4c-d). The size of the six alleles ranged 50-270 bp. Both cultivars showed homoallelic response for locus I except M_1 and S_1 which were heteroallelic. Similarly, at locus II, all the

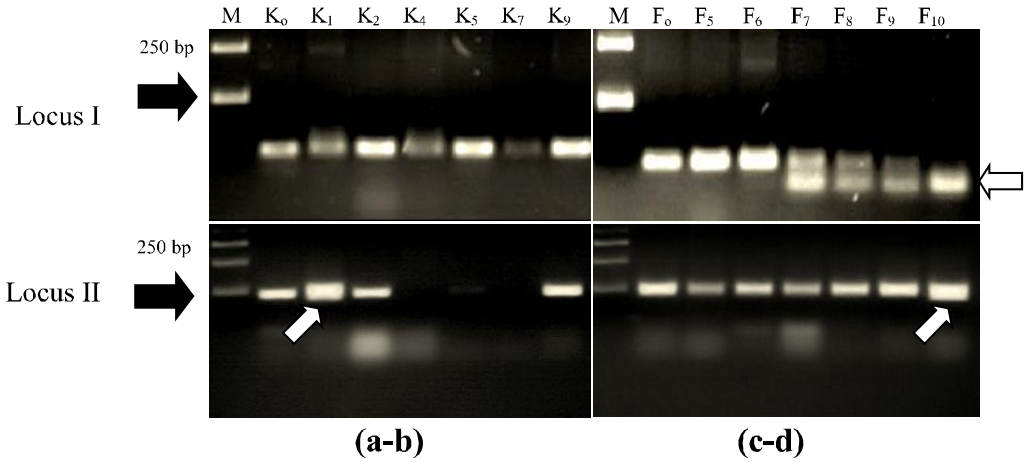

Figure 3a-d. Genotyping of mandarin polyploids cv. Kinnow (a-b) and cv. Feutrell's Early (c-d) through SSR. *M refer to I Kb ladder (Fermentas, Germany), Leaf DNA samples were as follows: lane 2 K_o and F_o are diploid and K_2-K_9 and F_5-F_{10} are different ploids amplified for polymorphism at locus I and same samples were subjected to DNA analysis using primer set two at locus II.*

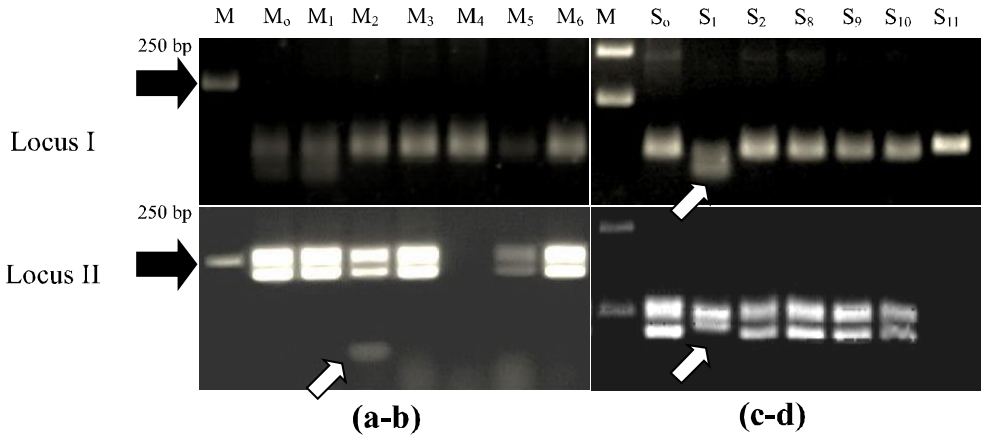

Figure 4a-d. Genotyping of sweet orange polyploids cv. Mosambi (a-b) and cv. Succari (c-d) through SSR. *M refer to I Kb ladder (Fermentas, Germany), Leaf DNA samples were as follows: lane 2 M_o and S_o diploid and M_1-M_6 and S_o-S_{11} are different ploids amplified for polymorphism at locus I and same samples were subjected to DNA analysis using primer set two amplifying locus II.*

genotypes of both the cultivars showed heteroallelic response except M_2 with a unique 3rd band (~50-100 bp) (white arrow in Fig. 3b) and S_1 with a unique 2nd band showing heterozygosity compared to rest of the genotypes (Fig. 4d). In M_4 and S_{11}, null alleles were observed at locus II which might be attributed to point mutations or deletions (Fig. 4b,d). Primer set I and II successfully amplified unique bands for characterization of Succari genotype S_1 and primer set II for Mosambi genotype (M_2). These different bands were found specie specific because these unique bands could not be detected in both cultivars Kinnow and Feutrell's Early as well as cultivar specific as both cvs. Mosambi and Succari belong to the same species.

DISCUSSION

Polyploids have been reported in *Citrus* cultivars like tetraploid Hongkong wild kumquat, tetraploid triphasia, tetraploids apomictic seedlings (Barrett and Hutchinson, 1978) and among progenies of Kinnow, Feutrell's Early, Mosambi, Kaghzi lime and grapefruit (Usman *et al.*, 2006, 2012). Different morphological markers has successfully been used for separating nucellar and zygotic *Citrus* seedlings among polyploid parents including characters like leaf margins and serration (Jaskani and Khan, 2000), ratio leaf length and width, petiole size (Donadio, 1981), petiole wing (Ballve *et al.*, 1997) and stem girth etc. (Khan *et al.*, 1992).

In several tree species, the number, size and frequency of stomata and number of chloroplasts have been a useful tool for early comparison of polyploids. Stomatal size was found directly related to ploidy level whereas number of stomata was inversely proportionate to ploidy in pear (Jia and Chen, 1985). Diploid Kinnow have a higher number of stomata per unit leaf area but the stomata were more developed in tetraploid leaves (Khan *et al.*, 1992). Number of chloroplasts was found directly proportional to ploidy of the plant. Stomatal studies and chromosome counting have been used as promising tools for preliminary characterization of diploids, polyploids and other taxa (Jaskani and Khan, 2000; Usman *et al*., 2012). However, it is further needed to explore the methods that can provide an accurate, reliable and efficient characterization of the polyploids. Molecular markers have been widely used in genetic characterization of plant germplasm. For example, to identify nucellar seedlings, to reduce number of trees maintained by identifying redundancies, to identify accessions for which the identification had been lost and to screen for pathogens (Krueger and Roose, 2003). Thomas *et al.* (1994) also reported identification and characterization of the genotypic polymorphism in *Citrus.* Several other workers have characterized zygotic and nucellar seedlings of a cross between Murcott Tangor (*C. reticulata* L. Blanco × *C. sinensis* L. Osbeck) and Pera sweet orange (Oliveira *et al.*, 2000), somatic recombinants of Clementine (*C. clementina* Hort. Ex. Tan.) (Breto *et al.*, 2001), interploid and intergeneric hybrids of Rangpur lime (*Citrus limonia* (L.) Raf.) and trifoliate orange (*Poncirus trifoliata* L.) as reported by Kijas *et al.* (1997). Markers like SSR have been successfully used for genetic characterization in several plant species (Cregan *et al.*, 1999), for detection of the genetic polymorphism and establishing phylogenetic relationships among *Citrus* and other genera (Pang *et al.*, 2003).

In the present studies, a variable percentage of triploids were observed as 9.95% in Kinnow, 8.85% in Mosambi, 7.4% in Succari and 7.01% in Feutrell's Early (Fig. 2b). Morphological studies of the produced germplasm indicated leaf lamina length and width and its ratio as a morphological marker for the successful identification of the spontaneous polyploids in Kinnow, Feutrell's Early and Succari cultivars of *Citrus* while in Mosambi angle of leaf petiole could be useful. These vegetative descriptors of ploids showed a direct proportion to the ploidy level as various leaf parameters studied increased with increase in the ploidy. A direct relationship of the stomatal size with ploidy in different citrus cultivars was observed as reported by Usman *et al.* (2006). The chloroplast number of tetraploid plants was almost twice that of diploid plants. Citrus cultivars under study showed wide differences for diploids, tetraploids and triploids. On the basis of the four stomatal parameters viz. number, width and length of stomata and number of chloroplasts, several plants were found triploid. Our findings are also in line with Jaskani and Khan (2000) who concluded that the number of the stomatal guard cells was directly proportional to ploidy level of the plants. Similar findings are reported by Usman *et al.* (2012) in grapefruit. However, one cannot fully rely on stomatal parameters for characterization of polyploids because differences in these parameters are cultivar dependent. Morphologically different *Citrus* ploids subjected to stomatal studies revealed that stomatal size and number of chloroplasts has been directly proportional to the ploidy level while number of stomata has been inversely proportionate. Climatic conditions also alter plant growth and in adverse climatic conditions wide differences can be observed in morphological parameters. Certain inconsistencies were observed in number of chloroplasts in sweet oranges which might be attributed to gene interaction or epistasis. Further characterization of these polyploid populations using Simple Sequence Repeat (SSR) analysis proved to be highly efficient.

Each of the primer sets used in this study produced one to three bands per genotype in diploid and polyploid plants and number of alleles per locus in both populations also varied from one to three. However, it was difficult to distinguish duplex versus triplex conditions because in some instances within a lane bands containing double or triple dose alleles appeared brighter than bands representing a single dose allele. In other case all bands had same intensity regardless of the allelic dosage. Diallelic condition was observed only in Mosambi and Succari population of polyploids for locus II and was less frequent compared with monoallelic behaviour commonly observed in Kinnow mandarin and Feutrell's Early spontaneous polyploids. On the basis of SSR analysis the polyploid accessions K_1, K_4, K_5, K_7 (Kinnow), F_7, F_8, F_{10} (Feutrell's Early), M_2, M_4 (Mosambi) and S_1, S_{11} (Succari) were found to be polymorphic. Sequencing of these SSR markers is suggested for future breeding and biotechnology applications.

***Conclusions*:** In these studies, the use of SSR-based loci genotyping followed by morphological and cytological screening of polyploids appeared to be a much improved strategy to identify seedling polyploids to develop germplasm bank by saving cost and reducing time.

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