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Genetic diversity and DNA fingerprinting of indigenous and exotic mandarin genotypes in India using SSR markers

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Abstract

Genetic variability and fingerprint profiles of 19 indigenous and exotic mandarin genotypes introduced from different parts of India and USA were determined using 60 SSR markers. Of the 57 SSR markers amplified, a total of 96 alleles were detected by 39 polymorphic SSR loci and maximum 5 alleles were amplified with an average of 2.46 alleles per primer pair. The CAT01 was the highly informative marker as it revealed maximum number of alleles (5), PIC value (0.75) and genetic diversity (0.79). Twenty six SSRs revealed specific/unique alleles and identified nine genotypes including all the hybrids. Across the genotypes, maximum number of alleles (83) was detected in Daisy hybrid and the percentage of polymorphic marker was maximum (80.32) in Nova hybrid. The markers with low number of alleles were able to differentiate the varieties with specific alleles. The higher average expected heterozygosity (35.6%) with in a mandarin group as compared to the average observed heterozygosity (27.2%) may be explained by selfing, which reduced the proportion of heterozygotes. The genotypes were classified in three clusters i.e. cluster- I, cluster- -II and cluster -III. All the indigenous genotypes (selections) were grouped in cluster -I and it had maximum genetic similarity coefficient. However, the exotic genotypes (hybrids) were grouped in cluster- II and cluster- II. Clustering was according to the breeding history of genotypes but independent to their geographic origin. The low observed heterozygosity frequency, PIC value, and number of alleles explained the narrow genetic base in the present set of mandarin genotypes.

Keywords: citrus; genetic; diversity; molecular marker; PIC; SSR.
Abbreviations: AFLP_amplified fragment length polymorphism; CTAB_cetyl trimethyl ammonium bromide; H_e_expected heterozygosity; H_0_observed heterozygosity; NTSYS_numerical taxonomy and multivariate analysis system; RAPD_randomly amplified polymorphic DNA; RFLP_restricted fragment length polymorphism; SSRs_simple sequence repeats; UPGMA _unweighted pair group method using arithmetic averages.

Introduction

Citrus is grown throughout the tropical and subtropical regions of the world. However, the best fruit quality is achieved under subtropical conditions. The center of origin and diversity of citrus and its related genera is generally considered to be Southeast Asia, especially East India, North Burma, and Southwest China, possibly ranging from Northeastern India eastward through the Malay Archipelago, North into China and Japan, and South to Australia (Gmitter and Hu, 1990 ; Soost and Roose, 1996). The genus *Citrus* belongs to the subtribe *Citrinae*, tribe *Citreaea*, subfamily *Aurantioideae* of the family *Rutaceae*. This genus may be further divided into two subgenera (*Citrus and Papeda*), based on leaf, flower and fruit properties.

In India, citrus is being cultivated on 1.04 million hectares with an annual production of 10.0 million tonnes (NHB, 2013). Among the citrus fruits in India, mandarin is placed at first position with respect to area and production followed by sweet oranges and limes. Mandarin is predominantly grown in Maharashtra, parts of Madhya Pradesh, Punjab and North Eastern Regions (NHB, 2013). The presence of 23 species, one subspecies and 68 varieties of citrus in Northeastern India gave special status to this region as treasure house of citrus germplasm (Sharma et al., 2004). Natural hybridization and the occurrence of spontaneous mutations are very common in citrus species. Cross pollination and the reported high percentage of zygotic twins have also resulted in greater variation in the plant types (Das et al., 2007). Mandarins exhibit more variation in characters than other citrus species (Reuther et al., 1967), and are the most phenotypically heterogeneous group in citrus (Moore, 2001). Therefore, the knowledge of genetic variation and relationship among different genotypes is an important consideration for strengthening the citrus industry in India. Morphological (Jaskani et al., 2006; Altaf and Khan, 2008 ; Sharma et al., 2004) and isozyme markers (Ashari et al., 1989; Elisirio et al., 1999; Rahman et al., 2001 ; Fang et al., 1997) have been employed in assessing the underlying genetic variation of citrus genotypes, however, these do not portray the reliable genetic relationships among the genotypes. This is due to the limited number of morphological and biochemical markers, low level of polymorphism, unknown genetic control of these traits, environmental interactions, stage specific identification and inadequate sampling of the genome (Yun et al., 2003). Therefore, a more precise system for identification of genotypes and for assessing the genetic variation in the existing germplasm is a fundamental requirement for establishing breeding programs and the registration of new cultivars in citrus. Various molecular markers such as

randomly amplified polymorphic DNA (RAPD), restricted fragment length polymorphism (RFLP) (Abkenar et al., 2004), amplified fragment length polymorphism (AFLP) (Campos et al., 2005 ; JinPing et al., 2009 and) and microsatellite markers (Barkley et al., 2006; Corazza-Nunes et al., 2002; Koehler et al., 2003, Kijas et al., 1995; Kacar et al., 2013 ; Ghanbari et al., 2009) have been used to evaluate phylogenetic relationships, characterize cultivars, assess diversity within citrus. Among them, microsatellites or simple sequence repeats (SSR) are highly polymorphic and co-dominant. Moreover, SSRs have greater power of discrimination than RFLP or RAPD markers because SSR genotypic data have the potential to provide unique allelic profiles and DNA fingerprints for establishing the precise genotypic identity (Yun et al., 2003). The existence of microsatellite sequences in citrus was first reported by Kijas et al. (1995). SSRs were used in citrus for the construction of genetic maps (Machado et al., 1996), for the assessment of genetic variability (Fang et al., 1997 ; Filho et al., 1998), for phylogenetic analysis (Kijas et al., 1997; Novelli et al., 2000) and for the identification of zygotic and nucellar seedlings (Kijas et al., 1995 ; Sanker et al., 2001). Simple Sequence repeats (SSRs) have been recognized as good sources of genetic markers in many plants including citrus (Akkaya et al., 1992; Kijas et al., 1997).

The department of Fruit Science of Punjab Agricultural University has the germplasm collection block of mandarin where different accessions introduced from other countries and all over India are being maintained. However, the level of genetic diversity of these collections was unknown. Therefore, the present study was conducted to characterize and to assess the genetic diversity in mandarin germplasm using SSR markers which is the basic need for any crop improvement program.

Results

Allele amplification in mandarin genotypes

In the present study, a total of sixty SSR primers were used (Supplementary table 1) to evaluate the genetic diversity of 19 mandarin cultivars. Three primer pairs (CCSM06, CCSM111 and AC01) failed to show any amplification thus revealing no bands (null allele) in all the genotypes. Of the 57 SSR markers amplified, 39 markers exhibited polymorphism (Table 1) and showed high levels of allelic diversity while remaining 18 were monomorphic. A total of 96 alleles were amplified by 39 polymorphic SSR loci and the number of alleles ranged from 1 to 5 with an average of 2.46 alleles per locus. One marker showed five alleles, two revealed four alleles, eleven revealed three alleles and for the remaining, twenty five amplified two alleles (Table 1).

Specific alleles for fingerprinting of genotypes

Specific alleles were identified in nine genotypes with SSR markers. Twenty six SSR primers were found to have higher discriminating potential for differentiation of the genotypes from other mandarin genotypes as they uncovered 28 specific/unique alleles in 9 genotypes (Table 2 and Fig. 1). These primers amplified more than one allele comprising one allele common in all genotypes. However, one allele was amplified in very few genotypes which differentiate these genotypes from other group. As the hybrids had the highest proportion of specific/unique alleles than varieties therefore, all the hybrids had been identified on the basis of specific alleles. Markers CCSM09 and CMS26 revealed specific alleles in Nagpur 51 and Coorg respectively though, both of these genotypes are selection. Marker CiBE4796 and ATC09 had specific alleles only for Nova hybrid, similarly CiBE0733 and 571 had specific alleles for Fremont, CiBE3298, CCSM170 and 495 had for Daisy and CCSM156, CTT01 and GT03 had specific alleles for W. Murcott only. Marker CiBE1116 and CiBE3397 had two genotype specific alleles while the remaining had one. The number (4) of varieties identified by CiBE3397 was more as it revealed unique alleles in Kinnow, Fremont, Daisy and Nova. The Unique DNA profiles of the all the genotypes could also be created using either four (CiBE3396, CiBE3966, CCSM70, CiBE6256, CiBE1116) or other set of microsatellite markers. Therefore, these SSR markers were enough to discriminate the 19 citrus genotypes.

Polymorphic Information Content and per cent polymorphism

Across all the genotypes a total of 1347 alleles were amplified by 57 SSR primers with an average of 70.89 alleles for each genotype (Table 3). The average of amplified fragments for polymorphic markers was 53.94 whereas for monomorphic, it was 16.95. The maximum number of alleles (83) was detected in Daisy whereas Kinnow showed least number (58) alleles. However, the percent of polymorphic markers was more in Kinnow (79.31) than Daisy (77.10). The percentage of polymorphic markers was maximum (80.32) in hybrid Nova followed by 80.00 per cent in Mudhkher which is a selection.

The percentage of polymorphism of the 39 polymorphic markers ranged from 50 to 100 (Table 1). Among these, 20 exhibited 100 per cent polymorphism, one was having 80 per cent, five with 66.66 per cent and the remaining 13 exhibited 50 per cent. Average polymorphism (%) of all the polymorphic primer pairs across all the genotypes was 78.11. The PIC value which is a measure of allelic diversity at a locus ranged from 0.10 (CCSM170) to 0.75 (CAT01) with an average value of 0.40. Twenty three SSR markers revealed PIC value of more than 0.40. Primer CAT01 amplified 5 alleles and had a highest PIC value of 0.75 followed by CCSM 70 and CSM4 in which 4 alleles were amplified and had PIC value of 0.72 and 0.63 respectively (Table 1). All the alleles amplified by CAT01 primer pairs on all the genotypes were well distinguishable.

Heterozygosity of SSR markers in mandarin group

In general, citrus are regarded as a group that shows high heterozygosity (Barrett and Rhodes 1976). Therefore, to examine the organization of genetic diversity within the mandarin group, observed heterozygosities were calculated (Table 1). Thus, the measure of the amount of heterozygosity across loci is used as a general indicator of the amount of genetic variability. The observed heterozygosity (H_0) ranged from 0.0 (CCSM 112) to 0.736 (AG14) with an average of 0.272 at the locus. However, the values of expected heterozygosity (H_e) ranged from 0.024 (CT19) to 0.736 (CAT01) with an average value of 0.356. The data revealed that average expected heterozygosity (H_e) with in a mandarin group was higher (35.6 %) as compared to the average observed heterozygosity (27.2%).

Genetic diversity in mandarin germplasm

Genetic diversity was ranged from 0.11 (CCSM170) to 0.79 (CAT01). The average value of genetic diversity across all the primers was 0.43 (Table 1).

Table 1. Number of alleles amplified, polymorphism (%), Polymorphic Information Content (PIC) value and genetic diversity and heterozygosity of SSR markers.

S. No.	SSR markers	Total no. of alleles	Monomorphic alleles	Polymorphic alleles	Polymorphism (%)	PIC	Genetic diversity	Heterozygosity Observed (H_0)	Expected (He)
1	CiBE0733	2	0	2	100	0.20	0.21	0.062	0.172
2	CiBE1116	3	0	3	100	0.53	0.56	0.124	0.406
3	CiBE3298	2	1	1	50	0.12	0.13	0.055	0.056
4	CiBE3397	3	1	2	66.66	0.40	0.42	0.081	0.200
5	CiBE3936	2	0	2	100	0.25	0.26	0.160	0.314
6	CiBE3966	2	1	1	50	0.49	0.52	0.650	0.440
7	CiBE4225	2	0	2	100	0.31	0.33	0.150	0.245
8	CIBE4721	2	1	1	50	0.38	0.40	0.310	0.262
9	CiBE4796	2	1	1	50	0.43	0.45	0.420	0.333
10	CiBE5156a	2	1	1	50	0.43	0.45	0.420	0.333
11	CiBE5171	3	1	2	66.66	0.29	0.31	0.096	0.124
12	CiBE5720	3	1	2	66.66	0.62	0.65	0.280	0.568
13	CiBE6006	2	1	1	50	0.18	0.19	0.105	0.102
14	CiBE6256	2	0	2	100	0.51	0.54	0.690	0.485
15	CCSM170	2	1	1	50	0.10	0.11	0.055	0.055
16	CCSM201	2	0	2	100	0.42	0.44	0.176	0.283
17	CCSM 09	3	0	3	100	0.41	0.43	0.270	0.500
18	CCSM 69	2	1	1	50	0.50	0.53	0.684	0.453
19	CCSM 70	4	0	4	100	0.72	0.76	0.478	0.712
20	CCSM 77	2	0	2	100	0.50	0.53	0.500	0.463
21	CCSM 112	2	0	2	100	0.38	0.40	0.000	0.362
22	CCSM 156	3	1	2	66.66	0.53	0.56	0.332	0.510
23	AG14	2	0	2	100	0.51	0.54	0.736	0.490
24	ATC09	2	0	2	100	0.36	0.37	0.055	0.319
25	CAT 01	5	1	4	80	0.75	0.79	0.316	0.726
26	CT19	2	1	1	50	0.14	0.15	0.066	0.024
27	CTT01	2	1	1	50	0.10	0.11	0.052	0.053
28	GT03	3	0	3	100	0.57	0.60	0.283	0.550
29	CMS4	4	0	4	100	0.63	0.66	0.221	0.600
30	CMS 16	2	1	1	50	0.51	0.54	0.720	0.463
31	CMS 26	2	0	2	100	0.51	0.54	0.610	0.463
32	CMS30	3	1	2	66.66	0.56	0.59	0.507	0.534
33	CMS31	3	0	3	100	0.50	0.53	0.095	0.450
34	CMS46	2	1	1	50	0.19	0.20	0.105	0.101
35	CMS47	2	0	2	100	0.22	0.23	0.220	0.443
36	458	2	0	2	100	0.32	0.34	0.150	0.230
37	571	2	1	1	50	0.12	0.13	0.055	0.055
38	506	3	0	3	100	0.58	0.61	0.202	0.483
39	495	3	0	3	100	0.57	0.60	0.127	0.527
	Total	96	19	77	3063.3	15.82	16.65	10.618	13.889
	Mean	2.46	0.48	1.97	78.11	0.40	0.43	0.272	0.356

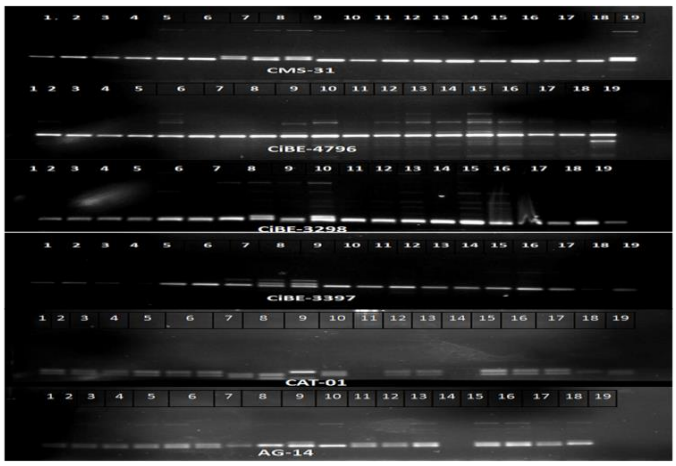

Fig 1. Agarose gel showing SSR amplification profile and specific alleles by different primer in different mandarin genotypes 1) Darjeeling mandarin 2) Coorg mandarin 3) Khasi mandarin 4) Nagpur mandarin 5) Mudkher mandarin 6) Nagpur Seedless mandarin 7) Kinnow, 8) Fremont 9) Daisy 10) W. Murcott 11) N-51 12) N-38 13) Clone- 11 14) CRS 4 15) N-43 16) N-34 17) N-28 18) N-4 and 19) Nova.

Table 2. Specific/unique alleles detected by SSR primers and identified mandarin genotypes.

S. No.	SSR markers	Total no. of alleles detected	Specific/unique alleles	*Genotype identified
1	CiBE0733	2	1	Fremont
2	CT19	2	1	Fremont
3	571	2	1	Fremont
4	CiBE3298	2	1	Daisy
5	CCSM170	2	1	Daisy
6	495	3	1	Daisy
7	CiBE4796	2	1	Nova
8	ATC09	2	1	Nova
9	CCSM 09	3	1	N-51
10	CCSM 156	3	1	W.Murcut
11	CTT01	2	1	W. Murcott
12	GT03	3	1	W. Murcott
13	CMS 26	2	1	Coorg
14	CiBE1116	3	1+1	Fremont and W. Murcott
15	AG14	2	1	Fremont and W. Murcott
16	CiBE6006	2	1	Fremont and Daisy
17	CMS46	2	1	Kinnow and Nova
18	CMS 16	2	1	Kinnow and Coorg
19	CiBE3397	3	1+1	Kinnow, Fremont, Daisy and Nova
20	CAT 01	5	1	Kinnow, Fremont and Daisy
21	CMS4	4	1	Kinnow, Fremont and Nova
22	CMS30	3	1	Kinnow, Daisy and Nova
23	CiBE4225	2	1	Fremont, Daisy and Nova
24	CCSM 70	4	1	Khasi, Nagpur and Fremont
25	CCSM 112	2	1	Fremont, Daisy and N-51
26	506	3	1	Daisy and W. Murcott

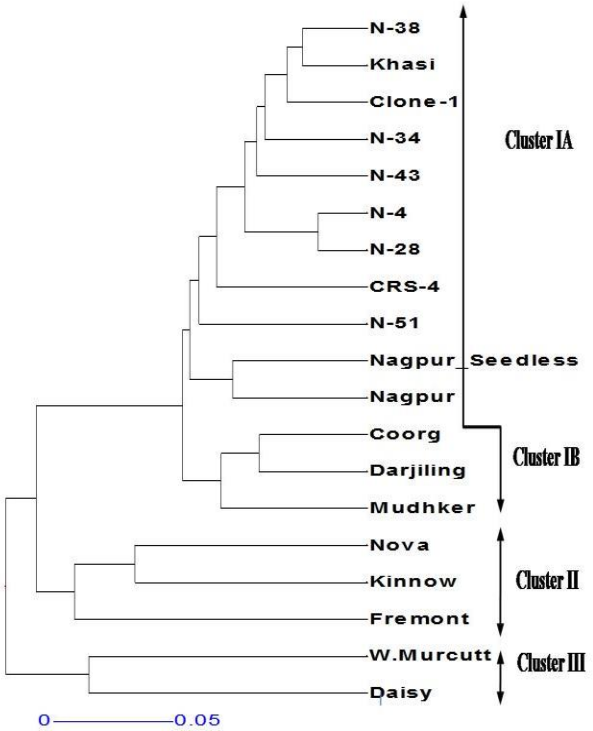

Fig 2. Dendrogram illustrating genetic relationship among 19 mandarin genotypes generated by UPGMA tree analysis. The cluster I was sub-divided into two sub cluster IA with eleven genotypes (N-38, Khasi, Clone 11, N-34, N-43,N-4, N-28,CRS-4,N-51,Nagpur Seedless and Nagpur) and IB with three genotypes (Coorg, Darjiling, and Mudhkher) respectively. In cluster II, three genotypes (Nova, Kinnow and Fremont) were clustered and cluster III comprised of two genotype (W. Murcutt and Daisy).

Table 3. Total number of alleles amplified in each of nineteen mandarin genotypes using sixty SSR markers.

S. No.	Genotypes	Number of amplified alleles		Total	Polymorphism (%)
		Monomorphic markers	Polymorphic markers		
1	Darjiling	15	47	62	75.80
2	Coorg	19	54	73	73.97
3	Khasi	17	57	74	77.02
4	Nagpur	18	58	76	76.31
5	Mudhkher	13	53	66	80.30
6	Nagpur Seedless	20	58	78	74.35
7	Kinnow	12	46	58	79.31
8	Fremont	20	59	79	74.68
9	Daisy	19	64	83	77.10
10	W. Murcott	20	56	76	73.68
11	Nagpur-51	18	58	76	76.31
12	Nagpur 38	16	55	71	77.46
13	Clone-11	19	51	70	72.85
14	CRS-4	13	49	62	79.03
15	Nagpur -43	20	56	76	73.68
16	Nagpur -34	18	54	72	75.00
17	Nagpur -28	17	49	66	74.24
18	Nagpur -4	16	52	68	76.47
19	Nova	12	49	61	80.32
	Total	**322**	**1025**	**1347**	**-**
	Mean	**16.94**	**53.94**	**70.89**	**76.09**

Table 4. Similarity coefficient based on DNA amplification of nineteen mandarin genotypes estimated by dice similarity coefficient.

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	0.91																	
3	0.88	0.90																
4	0.80	0.85	0.94															
5	0.89	0.87	0.93	0.86														
6	0.80	0.86	0.86	0.89	0.82													
7	0.71	0.76	0.73	0.74	0.73	0.77												
8	0.72	0.73	0.74	0.72	0.67	0.73	0.73											
9	0.64	0.68	0.71	0.70	0.66	0.64	0.66	0.72										
10	0.67	0.71	0.74	0.75	0.71	0.74	0.65	0.66	0.77									
11	0.86	0.89	0.88	0.83	0.82	0.84	0.70	0.76	0.73	0.74								
12	0.86	0.88	0.95	0.90	0.85	0.86	0.71	0.74	0.72	0.75	0.89							
13	0.87	0.89	0.92	0.88	0.86	0.83	0.71	0.73	0.72	0.74	0.91	0.94						
14	0.78	0.82	0.85	0.80	0.78	0.82	0.69	0.69	0.70	0.74	0.85	0.91	0.91					
15	0.82	0.86	0.90	0.87	0.80	0.80	0.69	0.70	0.68	0.71	0.85	0.92	0.90	0.84				
16	0.83	0.84	0.90	0.89	0.82	0.87	0.70	0.69	0.68	0.71	0.84	0.92	0.92	0.89	0.91			
17	0.84	0.86	0.90	0.85	0.83	0.82	0.65	0.71	0.70	0.71	0.83	0.92	0.91	0.88	0.88	0.91		
18	0.82	0.85	0.89	0.85	0.82	0.80	0.67	0.71	0.71	0.69	0.82	0.88	0.90	0.84	0.89	0.89	0.96	
19	0.67	0.73	0.77	0.77	0.69	0.76	0.81	0.79	0.67	0.57	0.73	0.77	0.75	0.72	0.73	0.74	0.74	0.78

The dendrogram (Fig. 2) depicting the genetic relationships as revealed by NTSYS-pc 2.02 classified the genotypes in to three major clusters (I, II and III). The cluster I was further sub-divided into two sub cluster IA and IB with eleven and three genotypes respectively. While three genotypes were clustered in cluster II and cluster III comprised of two genotypes. The indigenous mandarin genotypes form different geographical origins were grouped in cluster I whereas exotic genotypes (introduced from USA) were clustered in cluster II and III. The similarity coefficient based on DNA amplification of 19 mandarin genotypes using SSR primers was estimated by dice similarity coefficient (Table 4). The genotypes N-4 and N-28 showed the highest genetic similarity having similarity coefficient of 0.96 and were closely related. However, W. Murcott and Nova genotypes showed lowest (0.57) genetic similarity coefficient and these were genetically distinct from each other. All the genotypes in cluster I were clustered closely together as the genetic similarity coefficient ranged from 0.91 to 0.95. Whereas, it was 0.73 to 0.81 in cluster II and 0.77 in cluster III.

Discussion

Molecular markers are powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various citrus species (Barkley et al., 2006). The present study reveals the efficiency of SSR markers to assess genetic diversity and to distinguish the citrus genotypes at the species level. Out of the 60 SSR markers used in the present study, 3 revealed no amplification, 18 were monomorphic while 39 were polymorphic and amplified a total of 96 alleles. CAT01 has been reported as the highly polymorphic marker in citrus

Table 5. List of mandarin genotypes used to study phylogenetic relationship and genetic diversity by SSR markers.

S. No.	Common Name	Latin name	Parentage	Source
1	Darjiling	*Citrus reticulata* Blanco	Selection	Central Citrus Research Institute, Nagpur, Maharashtra, India
2	Coorg	*Citrus reticulata* Blanco	Selection	Central Citrus Research Institute
3	Khasi	*Citrus reticulata* Blanco	Selection	Central Citrus Research Institute
4	Nagpur	*Citrus reticulata* Blanco	Selection	Central Citrus Research Institute
5	Mudhkher	*Citrus reticulata* Blanco	Selection	Central Citrus Research Institute
6	Nagpur Seedless	*Citrus reticulate* Blanco	Nagpur Selection	Central Citrus Research Institute
7	Kinnow	*Citrus reticulata* Blanco	Willow leaf x King Mandarin	United States of America
8	Fremont	*Citrus reticulata* Blanco	Clementin mandarin x Ponkan Tangelo	United States of America
9	Daisy	*Citrus reticulata* Blanco	Fortune x Fremont	United States of America
10	W. Murcott	*Citrus reticulata* Blanco	Murcott and Unknown	United States of America
11	Nagpur-51	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute, Nagpur, Maharashtra, India
12	Nagpur 38	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute, Nagpur, Maharashtra, India
13	Clone-11	*Citrus reticulata* Blanco	Selection	Central Horticultural Experiment Station, Chettali Karnatka, India
14	CRS-4	*Citrus reticulata* Blanco	Selection	Assam Agricultural University,Tinsukia, Assam
15	Nagpur -43	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute, Nagpur, Maharashtra, India
16	Nagpur -34	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute Nagpur, Maharashtra, India
17	Nagpur -28	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute Nagpur, Maharashtra, India
18	Nagpur -4	*Citrus reticulata* Blanco	Nagpur Selection	Central Citrus Research Institute Nagpur, Maharashtra, India
19	Nova	*Citrus reticulata* Blanco	Clementin mandarin x Orlando Tangelo	United States of America

(Barkley et al., 2006; Jannati et al., (2009). In the present study, CAT 01 SSR marker is the most informative marker among the 60 SSR markers used as it revealed highest PIC value (0.75) and genetic diversity (0.79).

Nine genotypes could be identified on the basis of unique or specific alleles. The remaining genotypes could also be differentiated from one another on the basis of unique DNA profiles created by other polymorphic markers. Hence, the SSR markers used in the present study could precisely distinguish all the 19 genotypes from each other and thus, these SSR markers can be further used to differentiate the future genotypes from the existing ones. Number of alleles alone is not enough to differentiate the genotypes, however, it gives an indication about the level of genetic diversity in species or varieties (El-Mouei et al., 2011; Fang et al., 1997). Data revealed that the marker with more number of alleles has not proved its ability to differentiate cultivars through specific alleles. CiBE4225 and CCSM 112 amplified only two alleles but differentiated three cultivars each (Table 2) with specific alleles. The percentage of polymorphic markers and total number of alleles were maximum in hybrids Nova and Daisy respectively. It has been observed that marker CiBE5171 amplified three alleles and had PIC value of 0.29 while AG14 amplified two alleles and had PIC value of 0.51. Therefore, there seemed to be no strong correlation between the PIC value and the number of alleles amplified. In cluster analysis, it was observed that the genotypes from the different geographical regions were clustered across the subgroups without clear evidence of separation into subgroups by regions. All the genotypes which were selections were grouped in cluster I and showed the highest genetic similarity coefficient. This might be due to the fact of their common origin from the same parent. However, the hybrids Fremont and Nova had common female parent hence both were grouped in cluster II. Therefore, grouping of the genotypes as revealed by the cluster analysis was in congruent with the pedigree and breeding history of the genotypes. Furthermore, though, the genotypes revealed high genetic similarities with in a particular cluster but could be identified from each other using different primers. The co-dominant nature of microsatellites permits the detection of a high number of alleles/locus and contributes to higher levels of expected heterozygosity (H_e) than would be possible with dominant markers. The low observed heterozygosity frequency in mandarin may be explained by selfing, which reduced the proportion of heterozygotes. Lower level of heterozygosity in *P. trifoliata*, grapefruit (*C. paradisi*), sunki mandarin (*C. sunki*) and pummelo (*C. grandis*) has already been reported (Yaly et al., 2011). Species having low values of heterozygosity suggested the important role of self-fecundation within the origin and evolution of these species (Herrero et al., 1996). It has been observed that PIC values and number of alleles amplified by SSR markers were less as compared to reported earlier. Thus, the mandarin genotypes used in the present study is having narrow genetic base. The results are supported by Meral et al. (2011) who reported narrow genetic diversity in Satsuma mandarins clones suggesting that the observed morphological polymorphism within the group must be associated with somatic mutations which were not detected by SSR molecular markers. The analysis of 19 genotypes with 39 polymorphic microsatellite markers proved that most microsatellites were very informative and valuable in fingerprinting and evaluating genetic diversity of the present set of genotypes in mandarin.

Materials and Methods

Plant material

A total of 19 indigenous and exotic mandarin (*C reticulata* Blanco) accessions were used (Table 5) for genetic diversity studies. The hybrids used in the study were introduced from United States of America whereas other mandarin selections were introduced from different states of India. All the genotypes were grafted on rough lemon (*C. jambhiri* Lush.) rootstock and maintained at citrus gemplasm collection block of old orchard of Punjab Agricultural University, Ludhiana, Punjab (India) under standard package of practices recommended for citrus cultivation.

DNA extraction

Young leaves were collected from a single tree of each genotype. The leaves were wiped with ethanol and 100mg leaf tissue was used for DNA extraction. Genomic DNA was isolated using a standard Cetyl Trimethyl Ammonium Bromide (CTAB) procedure (Cheng et al., 2003). The purified DNA pellet was dissolved in 50µl of TE buffer and treated with RNAase. The DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and integrity of DNA was estimated by electrophoretic separation of DNA on 1% agarose gel. The final concentration of DNA was normalized to 15 ng/µl with sterile double-distilled water and both the stock and diluted portions were stored at -20°C until use.

Microsatellite amplifications

The DNA was amplified through polymerase chain reaction (PCR) using 60 SSR primer pairs (synthesized from Integrated DNA Technologies) previously described and used (Ahmad et al., 2003; Barkley et al., 2006; Ollitrault et al., 2010; Soriano et al., 2012; Yaly et al., 2011 ; Meral et al., 2011) for citrus germplasm characterization (Supplementary Table 1). PCR amplification of 20 µl total volume was performed in 2.0 µl of 10X PCR buffer, 2.5µl of 1mM dNTPs, 1.25 µl of each of forward and reverse primer (5 µM), 0.25 µl of Taq polymerase (5 units/µl of Promega, USA), 4.0 µl of DNA (15ng) and distilled de-ionized water using an Eppendorf thermal cycler. The PCR profile consisted of initial denaturation at 94°C for 3 min and subsequent 35 cycles each with denaturation at 94°C for 30s, primer annealing at 48-57°C for 1min and primer extension at 72°C for 1min. Final extension step was performed at 72°C for 7 min. Annealing temperature was modified to optimize the reaction conditions for individual primers. PCR products were stored at 4°C before analysis. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1X TBE (45 mM Tris-borate1 mM EDTA) and 0.5 µg/ml aqueous solution of ethidium bromide. The agarose gel was run at a constant voltage of 100V for 2–3 h in 0.5× TBE buffer. Gels were visualized under UV light and photographed using photo documentation system. The repeatability of the markers was verified in the whole collection and all null alleles were confirmed by a second amplification.

Data collection and analysis

SSR alleles were scored for the presence (1) and absence (0) of the SSR bands. Bands that were either diffused or those that were too difficult to score were considered as missing (9). Scoring was performed by visual identification. Specific alleles or unique alleles among total alleles amplified were identified in each genotype with each primer pair. Polymorphism information content (PIC) for each SSR marker was determined as per the procedure outlined by Senior et al. (1998).

$$PIC = 1-\sum_{i=1}^{n} (P_{ij})^2$$

Where; *Pij* is the frequency of j^{th} allele in i^{th} primer and summation extends over 'n' patterns.

Genetic similarity coefficients between various genotypes (in pair-wise comparisons) were calculated from the SSR data matrix using dice coefficient and the resulting genetic similarity matrix was analyzed using NTSYS-PC version 2.02 to produce an agglomerative heirarchical classification (Rohlf, 1989) by employing Unweighted Pair Group Method using Arithmatic Averages (UPGMA). For estimating the similarity matrix, null alleles (no SSR alalle in a given *citrus* genotype) were treated as missing data to reduce the biased genetic or similarity measures (Warburton and Crossa, 2000). Genetic diversity (GD) was calculated according to the following formula of Nei (1987).

GD= n (1- p2)/ (n-1)

Where; (*n*) is the number of samples and (*p*) is the frequency of one allele.

Observed heterozygosity (H_0) and expected heterozygosity (H_e) were also calculated (Marshall et al., 1998)

Where; H_0 = number of hetrozygous individuals per number scored Expected heterozygosity (H_e) was calculated by the following formula: $He = 1 - \Sigma pi^2$

Conclusion

The genotypes categorized in different clusters can be used by breeders to develop new cultivars. Though the genotypes showed high genetic similarity but can be identified by revealing specific alleles by some of the markers. Therefore, the set of microsatellites in the present study were successful in fingerprinting and evaluating genetic diversity in the mandarin genotypes which will be of great utility for protection and breeding of citrus germplasm.

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References

Abkenar AA, Isshiki S, Tashiro Y (2004) Phylogenetic relationships in the "true citrus fruit trees" revealed by PCR-RFLP analysis of cpDNA. Sci Hort. 102: 233-242.

Ahmad R, Struss D, Southwick SM (2003) Development and characterization of micro satellite markers in citrus. J Amer Soc Hort Sci. 128 : 584-590.

Akkaya MS, Bhagwat AA, Cregan PB (1992) Length polymorphisms of simple sequence repeat DNA in soybean. Genetics. 132: 1131-1139.

Altaf N, Khan AR (2008) Variation within Kinnow (*Citrus reticulata* Blanco) and Rough Lemon (*Citrus jambhiri* Lush.). Pak J Bot. 40: 589-598.

Ashari S, Aspinall D, Sedgley M (1989) Identification and investigation of relationships of mandarin types using isozyme analysis. Sci Hort. 40: 305-315.

Barkley NA, Roose ML, Krueger RR, Federici CT (2006) Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theor Appl Genet. 112: 1519-1531.

Barrett HC, Rhodes AM (1976) A numerical study of affinity relationships in cultivated citrus and its close relatives. Syst Botany. 1: 105-136.

Campos ET, Espinosa MAG, Warburton ML, Varela AM, Monter AV (2005) Characterization of mandarin (*Citrus spp*.) using morphological and AFLP markers. For Docum Elect (ISO). 30: 687-693.

Cheng Yun-jiang, Guo Wen-wu, Yi Hua-lin, Pang Xiao-min, Deng xiuxin (2003) An efficient protocol for genomic DNA extraction from *Citrus* species. Plant Mol Biol Report. 21: 177a–177g.

Corazza-Nunes, Machado MJ, Nunes MA, Cristofani WMC, Targon M, MLPN (2002) Assessment of genetic variability in grapefruits (*Citrus paradisi* Macf.) and pummelos (*Citrus maxima* Burm. Merr.) using RAPD and SSR markers. Euphytica. 126: 169-176.

Das A, Mondal B, Sarkar J, Chaudhuri S (2007) Occurrence of zygotic twin seedlings in mandarin orange plants of the northeastern Himalayan region. Curr Sci. 92: 1488-1489.

Elisirio PJ, Justo EM, Leitio JM (1999) Identification of mandarin hybrids by isozyme and RAPD analysis. Sci Hort. 81: 287-299.

El-Mouei R, Choumane Wand Dway F (2011) Characterization and estimation of genetic diversity in *Citrus* rootstocks. Int J Agri Biol. 13: 571-575.

Fang DQML, Roose, Krueger RR, Federici CT (1997) Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and Inter-Simple Sequence Repeat Markers. Theor Appl Genet. 95: 211-219.

Filho HDC, MA, Targon MLPN, Moreira MCPQDG, Pompeu J (1998) Analysis of the genetic diversity among mandarins (*Citrus* spp.) using RAPD markers. Euphytica. 102 (1): 133-139.

Ghanbari A, Jelodar NB, Rahiman H (2009) Studying of genetic diversity in Satsuma (*Citrus unshiu*) mandarin utilizing microsatellite markers. Int J Agri Res. 4 (2): 88-96.

Gmitter JFG, Hu X (1990) The possible role of Yunnan, China in the origin of contemporary Citrus species (Rutaceae). Econ Bot. 4: 267-277.

Herrero R, Asins MJ, Carbonell EA, Navarro L (1996) Genetic diversity in the orange subfamily Aurantioideae, Intraspecies and Intragenus genetic variability. Theor Appl Genet. 92 (5): 599-609.

Jannati M, Fotouhi R, Pourjan Abad A, Zivar Salehi (2009) Genetic diversity analysis of Iranian citrus varieties using micro satellite (SSR) based markers. J Hort Forest. 1: 120-125.

Jaskani MJ, Abbas H, Khan MM, Shahid U, Hussain Z (2006) Morphological description of three potential citrus rootstocks. Pak J Bot. 38: 311-317.

JinPing X, LiGeng C, Ming B, HaiLin L, WeiQi Y (2009) Identification of AFLP fragments linked to seedlessness in Ponkan mandarin (*Citrus reticulata* Blanco) and conversion to SCAR markers. Sci Hort. 121: 505-510.

Kacar Yildiz, Aydin Uzun, Ilknur Polat, Turgut Yesiloglu , Bilge Yilmaz , Osman Gulsen, Onder Tuzcu 1, Muge Kamiloglu , Senay Kurt, Ubeyit Seday (2013) Molecular characterization and genetic diversity analysis of mandarin genotypes by SSR and SRAP markers J Food Agric Environ. 11 (1): 516 -521.

Kijas JMH, Fowler JCS, Thomas MR (1995) An evaluation of sequence tagged microsatellite site markers for genetic analysis within citrus and related species. Genome. 38: 349-55.

Kijas JMH, Thomas MR, Fowler JCS, Roose ML (1997) Integration of trinucleotide microsatellites into a linkage map of citrus. Theor Appl Genet. 94: 701-806.

Koehler SP, Dornelles ACL, Freitas LB (2003) Characterization of mandarin citrus germplasm from Southern Brazil by morphological and molecular analyses. Pesq Agropec Bras. 38: 797-806.

Machado MA, Filho HDC, Targon MLPN, Pompeu JJ (1996) Genetic relationship of mediterranean mandarins (*Citrus deliciosa*) using RAPD markers. Euphytica. 92: 321-326.

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol. 7: 639-655

Meral N, Tuzcu O, Yeilolu T, Kacar Y A, Yıldırım B, Boncuk M, Cimen B (2011) Molecular diversification and preliminary evaluations of some Satsuma selections performance under mediterranean conditions. Afri J Biotech. 10: 4347-4357.

Moore GA (2001) Oranges and lemons: clues to the taxonomy of Citrus from molecular markers. Trends Genet. 17: 536-540.

Nei M (1987) Molecular Evolutionary Genetics New York: Columbia University Press

NHB (2013) Fruit Production Database. National Horticulture Board, New Delhi, India (*available online: www.nhb.in*).

Novelli VM, Cristofani M, Souza AA, Machado MA (2006) Development and characterization of polymorphic simple sequence repeats (SSRs) in sweet orange (*Citrus sinensis* L. Osbeck). Genet Mol Biol. 29: 90-96.

Ollitrault F, Terol J, Pina JA, Navarro L, Talon M, Ollitrault P (2010) Development of SSR markers from *Citrus clementina* (*Rutaceae*) BAC end sequence and inter-specific transferability in citrus. Amer J Bot. e124-e129.

Rahman M M, Nito N, Isshiki S (2001) Cultivar identification of 'Yuzu' (*Citrus junos* Sieb. ex Tanaka) and related acid citrus by leaf isozymes. Sci Hort. 60: 17-22.

Reuther W, Batchelor LD, Webber HJ (1967) The Citrus Industry. 1: 500-505.

Rohlf FJ (1989) NTSYS-pc Numerical Taxonomy and Multivariate Analysis System, version *2.02*. Sarauket, NY: Exeter publication.

Sanker AA, Moore GA (2001) Evaluation of Inter- Simple Sequence repeat analysis for mapping in citrus and extension of the genetic linkage map. Theor Appl Genet. 102 (2): 206-214.

Senior ML, Murphy JP, Goodman MM, Stuber CW (1998) Utility of SSRs for determination genetic similarities and relationship in maize using an agarose gel system. Crop Sci. 38: 1088-1098.

Sharma BD, Hore DK, Gupta SG (2004) Genetic resources of Citrus of north-eastern India and their potential use. Genet Resour Crop Evol. 51: 411-418.

Soost RK, Roose, ML (1996) Citrus. In: Jsss Janick and JN Moore (Eds.), *Fruit Breeding*, Vol. I: Tree and Tropical Fruits. John Wiley and Sons Inc., New York, Chiche- ster, Brisbane, Toronto, Singapore, pp. 257-323.

Soriano L, Filho F de AAM, Camargo L EA, Cristofani-Yaly M, Latado R R, Pacheco C de A, Alves de Azevedo F, Mendes B M J (2012)Regeneration and characterization of somatic hybrids combining sweet orange and mandarin/mandarin hybrid cultivars for citrus scion improvement. Plant Cell Tiss Organ Cult. 111:385–392

Warburton M, Crossa J (2000) Data analysis in the CIMMYT Applied Biotechnology Centre for Fingerprinting and Genetic Diversity Analysis. CIMMYT. Texcoco, Mexico.

Yaly MC, Novelli VM, Bastianel M, Machado MA (2011) Transferability and level of heterozygosity of microsatellite markers in citrus species. Plant Mol Biol Rep. 29: 418-423.

Yun-Jiang Cheng, Wen-Wu Guo, Hua-Lin Yi, Xiao-Min Pang, Xiuxin Deng (2003) An Efficient protocol for genomic DNA extraction from citrus species. Plant Mol Bio Rep. 21: 177.