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Assessing genetic diversity in Indian pummelo collections utilizing quantitative traits and simple sequence repeat markers (SSRs)

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ABSTRACT

The study was to characterize 20 Indian pummelo genotypes based on quantitative traits and microsatellite (SSR) markers. Among the fruit quantitative traits, the highest coefficient of variation (CV) was recognized for segment numbers (16.64%), followed by core diameter (13.93%), juice recovery (11.20%) and fruit weight (10.88%). In comparison, it was low for TSS (1.18%) and ascorbic acid (1.83%). The result of the PCA showed that six out of the nineteen principal component axis (PCA) had Eigen-values greater than one, and all together accounted for 63.47 of the total variability. Microsatellite based study revealed that PIC value ranged from 0.56-0.18, highest from TC26 (0.56) and lowest from AC01 (0.18). The gene diversity ranged from 0 to 1. The heterozygosity ranged from AC01 (0.16) to TC26 (0.47), with an average of 0.31 per locus. The results also indicated that all twenty pummelo genotypes were demarcated into two major clusters, A and B, according to the UPGMA method.

Keywords: *Citrus maxima* (J. Burm.) Merr., quantitative traits, microsatellite (SSR) markers

INTRODUCTION

Citrus is the most produced fruit in the world with over the 124 million tons of production (FAO, 6). Among the different citrus species Pummelo or Shaddock is considered to be one of three true citrus species together with citron (C*itrus medica*) and mandarin (*C. reticulata*) based on karyotype analysis (Hynniewta *et al*., 9). This species is also a progenitor of the grapefruit (C. *paradisi*) and the tangelo among other modern citrus hybrids. In recent years, the demand for pummelo has increased mainly in warm areas of the world where other sweet citrus fruits cannot be grown. Furthermore, the pummelo is now widespread in Bangladesh, Chile, Cambodia, India, Indonesia, Japan, Malaysia, Thailand and Vietnam. In India, different *Citrus* species are grown, however, the vast genetic diversity of wild and semi-wild citrus germplasm has modestly been used for improvement programmes due to lack of their characterization, because of the wide gaps in the knowledge of useful characters of various citrus species and varieties (Sharma *et a*., 16). The duplicates within a gene bank collection are usually unintentional and undesired, which can occur due to exchange of accessions between gene banks or acquisition of the same accession *i.e.* a cultivar by several gene banks or due to safety-duplication. Information on genetic diversity and phylogeny of pummelo genotypes can improve the efficiency of germplasm characterization and its use in breeding programs. Moreover, genetic diversity within and among populations is the backbone of conservation of plant genetic resources for both present and future uses. Morphological traits are among the first markers used in germplasm management (Gitonga *et al*., 7), besides having significance of the existence in differentiating between subspecies or varieties within taxa (Paganova, 11). Biochemical markers are another important group of marker, they are co-dominant, easy to use and cost effective. However, they are less in number; they detect less polymorphism, and are affected by various extraction methodologies, plant tissues and different plant growth stages. Several authors have investigated and characterized different selections of citrus plants, in order to increase the number of genotypes with potential to be used in breeding programs or to be released as new varieties. Molecular markers are nucleotide sequences, and can be investigated through the polymorphism present between the nucleotide sequences of different individuals. Among the different molecular markers, simple sequence repeats (SSR) is the powerful molecular marker for assessing genetic variation in plant due to its low cost, high polymorphism and co-dominant inheritance (Barkley *et al*., 1).

The analysis of genetic diversity among the genotypes provide the base for pummelo breeding and resource conservation. In view of the problems with respect to safety of germplasm maintained in gene banks, it is necessary to identify duplicates and to concentrate on unique accessions for

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easy management. Hence, the present study was undertaken to assess the variability within existing pummelo genotypes, using quanititative traits and microsatellite (SSR) markers.

MATERIALS AND METHODS

Out of twenty pummelo genotypes selected, eighteen (PS-1 to PS-18) were collected from NBPGR regional station, Bhowali, Uttrakhand and other pummelo growing areas of the country. Two pummelos (PM-3 and PM-4) selected from Division of Fruits and Horticultural Technology, IARI, New Delhi as spontaneous mutant. The budded plants of selected clones and mutant were transplanted in citrus evaluation block of Division of Fruits and Horticultural Technology, IARI at 5 m x 5 m distance. Trees were given recommended doses of nutrients. Pomological characterization of 20 pummelo germplasm was carried out using different quantitative traits viz., leaf size, leaf area, lamina wing ratio and petiole area as well fruit quality traits from 2017-2019 on 14 years old trees. Ten randomly selected fruits from each genotype from all directions were harvested at physiological maturity for recording physical fruit parameters such as fruit weight, size, juice content peel thickness, segment and number of seeds. Total soluble solids (TSS) were determined using digital refractometer (ATAGO PAL-3), however, titratable acidity was estimated according to the method described by Rangana (13). The ascorbic acid content in fruit juice was estimated by iodometric titration (Silva *et al*., 17).

For genomic study, young, tender and healthy leaves (250 g) from new flush were collected from the selected tree of each genotype and wrapped in polyethylene bags with proper labelling and placed in ice box. On reaching the laboratory, leaves were washed under tap water and cleaned with tissue paper. The midribs and thick veins of the leaves were removed. Samples were wrapped in aluminium foil, labelled properly and stored at -20ºC temperature till DNA extraction. Total genomic DNA was extracted from previously collected leaves of all the twenty pummelo accessions by Cetyl trimethyl ammonium bromide (CTAB, Ameresco) method (Doyle and Doyle, 4). Purification and quantification of DNA was done as per the method suggested by Shareefa (15). The list of the amplified primers used along with their sequence and other details are given in Table 1.

For fruit and leaf quality parameters, three replications per treatment were included. Data were analysed in a one-way ANOVA in SAS 9.3 (SAS Institute Cary, NS, USA). Cluster analysis was done by unweighted pair-group method with arithmetic mean (UPGMA) Jaccard's similarity coefficient. Data were analyzed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc version 2.1) software package (Rohlf, 14). Principal components analysis (PCA) of all accessions using 11 quantitative traits was performed by NCSS 2007

Table 1. List of the amplified primers, sequence and annealing temperature.

S. No.	SSR Primer	Forward Primer Sequence	Reverse Primer Sequence	T_m(°C)	T_a(°C)
1.	AC01	TTTGACATCAACATAAAACAAGAAA	TTTTAAAATCCCTGACCAGA	56.0 52.3	50
2.	CAC23	ATCACAATTACTAGCAGCGCC	TTGCCATTGTAGCATGTTGG	55.4 53.8	51
3.	CAG01	AACACTCGCACCAAATCCTC	TAAATGGCAACCCCAGCTTTG	58.4 59.4	56
4.	CCSM-6	ATCTGTGTGAGGACTGAA	CCTCTATTAATGTGCCTG	51.6 51.6	49
5.	CCSM18	GTGATTGCTGGTGTCGTT	AACAGTTGATGAAGAGGAAG	53.9 54.3	51
6.	CgEMS-1	ACCCAAAATTGTCTCTTGCC	TCCCGATTTGGTGGTAAAAA	56.4 54.3	52
7.	CgEMS-45	GGAGCCTCTCTTCACACTCG	CGTTCTCTTCTTCGGCAGTC	62.5 60.5	58
8.	TAA45	GCACCTTTTATACCTGACTCGG	TTCAGCATTTGAGTTGGTTACG	55.4 53.7	51
9.	TC26	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA	60.5 58.4	56

(T_m=Melting Temperature, and T_a= Annealing Temperature)

v 07.1.18 (Hintez, 8). The principal component score with eigen values >1 were used as new variable for cluster analysis.

RESULTS AND DISCUSSION

The study of analysis of variance for different quantitative traits revealed the variability among all the quantitative characters of leaves, petiole and fruits. The analysis of variance for different quantitative traits of fruits (Table 2) indicated the highest coefficient of variation (CV) for segment numbers (16.64%) followed by core diameter (13.93 %), juice content (13.27%) and juice percent (11.20 %), fruit weight (10.88%) and peel thickness (9.11 %), while very low CV was observed for TSS (1.18%) and ascorbic acid (1.83%). Amongst the leaf quantitative traits highest coefficient of variation (CV) was obtained for lamina wing ratio (41.23%) followed by petiole area (28.86%) and leaf area (17.79%), whereas the lower CV was noted for leaf width (11.23%). The difference in individual genotype might be contributed due to mutations, and cross pollination. Das *et al*. (2) found that, the common occurrence of zygotic twins in Himalayan mandarin varieties might be the possible cause for the variation as observed in our study. The bud sport mutations, introduction and trials of materials in location different from its original habitat add the differences among the studied population (Dorji and Yapwattanaphun, 3). Susandarini *et al.* (19) also found huge variation in Malaysian pummelos. While, genetic diversity study of pummelo landraces of Indian origin, established that many superior pummelo clones are managed by local farming communities inhabiting various agro-eco-niches as on farm conservation (Singh *et al*., 18).

The PCA was used to determine the extent of the variation and percentage similarity within the pummelo genotypes (Table 3). Eigen-values and factor scores obtained from PCA were used to determine the relative discriminative power of the axis and their associated characters. The result of the PCA showed that six out of the nineteen principal component axis (PCA) had Eigen-values greater than one, and all together accounted for 63.47% of the total variability. The first PCA 1 accounted for 30.60 of the total variation, the second PCA 2 accounted for 14.61% of the total variations and third PCA 3 accounted for 12.10% of the total variations. The cumulative per cent of variance varied from 30.60 to 80.62% for the PCA which had Eigen value more than 1. The relative discriminating capacity of the PCA was shown by their Eigen-values. The PCA 1 had the highest discriminating power as revealed by

Table 2. Analysis of variance for different quantitative traits of pummelo genotypes.

Trait	Range	Population mean	R^2	CV (%)	F value
Fruit weight (g)	277.00-997.83	595.49	0.93	10.88	30.49
Fruit length (mm)	79.36-130.17	105.98	0.44	4.42	35.36
Fruit diameter (mm)	84.74-132.20	11.78	0.92	4.33	26.85
Peel thickness (mm)	7.59-19.59	12.34	0.91	9.11	21.70
Core diameter (mm)	9.97-44.79	25.81	0.91	13.93	24.05
Segment (No.)	0-150.00	70.77	0.94	16.64	35.14
Seeds/ fruit	13.33-20.33	15.27	0.77	6.92	6.93
Juice (ml)	60.67-228.00	126.79	0.92	13.27	26.20
Juice (%)	10.58-39.51	21.93	0.92	11.20	25.59
TSS (°B)	7.00-13.63	8.88	0.99	1.18	403.23
Acidity (%)	0.38-0.94	0.62	0.97	5.61	78.87
TSS/acid ratio	7.22-23.38	15.30	0.96	7.02	50.24
Ascorbic acid (mg/100 ml juice)	30.29-97.75	50.21	1.00	1.83	1002.66
Leaf length (cm)	6.66-10.48	9.19	0.69	12.26	4.75
Leaf width (cm)	4.77-6.40	5.68	0.50	11.23	2.11
Leaf length/ width ratio	1.40-2.12	1.62	0.50	13.83	2.14
Leaf area (cm^2)	30.05-58.54	46.40	0.63	17.79	3.73
Petiole area (cm^2)	0.86-8.31	9.68	0.78	28.86	7.56
Lamina wing ratio	5.79-59.87	16.94	0.84	41.23	11.16

Table 3. Principle component analysis of pummelo genotypes using quantitative traits.

PCA	Trait	Eigen-Value	% Variance	% Cumulative Variance
PCA 1	Fruit weight (g)	5.81	30.60	30.60
PCA 2	Fruit length (mm)	2.78	14.61	45.21
PCA 3	Fruit diameter (mm)	2.30	12.10	57.31
PCA 4	Peel thickness (mm)	1.58	8.32	65.64
PCA 5	Core diameter (mm)	1.52	8.01	73.65
PCA 6	Segment (numbers)	1.32	6.98	80.62
PCA 7	Seeds/fruit	0.90	4.74	85.37
PCA 8	Juice (ml)	0.71	3.79	89.15
PCA 9	Juice (%)	0.62	3.29	92.44
PCA 10	TSS (°B)	0.43	2.27	94.72
PCA 11	Acidity (%)	0.32	1.70	96.43
PCA 12	TSS/acid ratio	0.23	1.24	97.68
PCA 13	Ascorbic acid (mg/100 ml juice)	0.15	0.83	98.51
PCA 14	Leaf length (cm)	0.14	0.79	99.30
PCA 15	Leaf width (cm)	0.07	0.39	99.69
PCA 16	Leaf length/width ratio	0.02	0.12	99.81
PCA 17	Leaf area (cm^2)	0.02	0.11	99.93
PCA 18	Petiole area (cm^2)	0.01	0.06	99.99
PCA19	Lamina wing ratio	0.01	0.01	100.00

its highest Eigen-value of 5.81 followed by PCA 2 with Eigen value of 2.78. PCA has successfully found linear combinations of the different quantitative traits, which separated out different clusters of pummelo genotypes. The genotypes were classified into four distinct cluster groups (Fig.1). Therefore, the Principal component analysis identified that the cumulative variation explained by first six components accounted over 63.47%variations, revealing great quantitative traits variability, a high genetic diversity between pummelo genotypes. This suggested that these traits had significant contribution in pummelo diversity. Similar findings have also been reported in lime (Dubey *et al*., 5) and pummelo (Paudyal and Haq, 12).

Amongst the 54 primers selected to characterize pummelo genotypes, 9 primers selected for the analysis of generated polymorphic allelic pattern in 20 pummelo genotypes. The SSR primer TC26 produced maximum number of 3 different alleles, primers CAG01 and CCSM-6 produced 2 different alleles each, and the rest six primers could produce only 1 allele (Table 4). The amplified fragments ranged from 110-250 bp. The PIC was calculated for each primers and it was ranged from 0.56-0.18. The highest PIC was calculated from TC26 (0.56) and lowest PIC from AC01 (0.18). The gene diversity was ranged from 0 to 1. The heterozygosity ranged from AC01 (0.16) to TC26 (0.47) with an average 0.31 per locus. Wang *et al*. (20) studied the genetic diversity of pummelo germplasms of Sichuan Basin inferred from SSR Markers, and noted the obvious differences of genetic diversity among different pummelo varieties groups.

A dendrogram of the genetic relationships among pummelo genotypes was drawn according to the UPGMA method (Fig.2). The results indicated that all twenty pummelo genotypes were demarcated into two major clusters A and B (Fig. 2). The major cluster A consist of four genotypes, which was further divided into two sub clusters AI and AII. Sub cluster AI consist of two genotypes (PS-4 and PS-5) and both the genotypes showed 100 per cent similarity, whereas the sub cluster AII consist of PS-15 and PS-18. The major cluster B consist of 16 genotypes, which was further divided into two sub cluster BI and BII. The sub cluster BI consist of 13 genotypes, which was further divided into sub-sub cluster BI.a and BI.b. The sub-sub cluster BI.a includes 8 genotypes, whereas sub-sub cluster BI.b consists 5 genotypes. Further in sub-sub cluster BI.b with genotype PS-8 forms an outgroup. The sub cluster BII consist of three genotypes, which was again divided into sub sub cluster BII.a (PS-6) and BII.b (PS-10 and PS-12). Similarly, the genetic diversity analysis of Iranian citrus varieties using micro satellite (SSR) based

Table 4. Simple sequence repeat (SSR) primers, allelic loci, size range, values of polymorphic information content (PIC), gene diversity and heterozygosity (He).

S. No.	SSR Primer	No. of alleles	Size range (bp)	PIC	Gene diversity	He
1	AC01	1	160	0.18	0.00	0.16
2	CAC23	1	250	0.42	0.00	0.33
3	CAG01	2	110-120	0.50	1.00	0.38
4	CCSM-6	2	210-230	0.50	1.00	0.38
5	CCSM18	1	200	0.38	0.00	0.30
6	CgEMS-1	1	180	0.32	0.00	0.27
7	CgEMS-45	1	200	0.26	0.00	0.22
8	TAA45	1	190	0.38	0.00	0.30
9	TC26	3	140	0.56	1.00	0.47

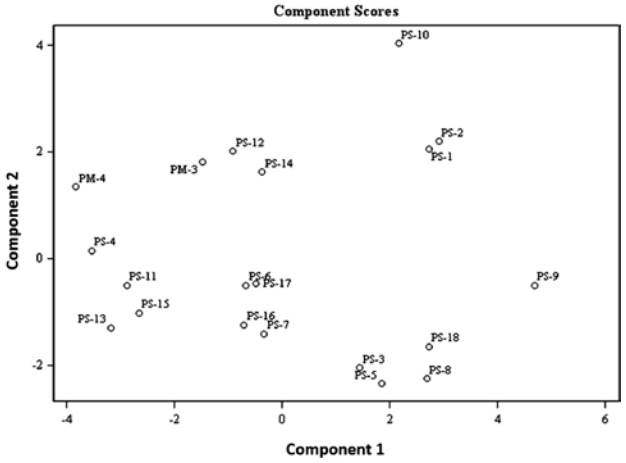

Fig. 1. A principal component analysis (PCA) scatter plot of pummelo genotypes using nineteen quantitative parameters.

markers revealed Cluster analysis with SSR markers resulted in 2 cluster groups (Jannati *et al*., 10). Based on results it can be concluded that wide range of variation in physico-chemical parameters of pummelo fruits showed the great possibility of individual plant selection based on these characters for future genetic improvement programme. Furthermore, fruit weight, fruit length, peel thickness, core diameter, number of segment and seeds can contribute a high genetic diversity between pummelo genotypes.

AUTHORS' CONTRIBUTION

Conceptualization of research (Dubey A.K.); Designing of the experiments (Dubey, A. K. and Sharma, R. M.); Execution of field/lab experiments and data collection (Kholia, A); Analysis of data and interpretation (Sharma, R. M. and Sharma, N); Preparation of the manuscript (Dubey, A. K., Sharma, R. M. and Sharma, N).

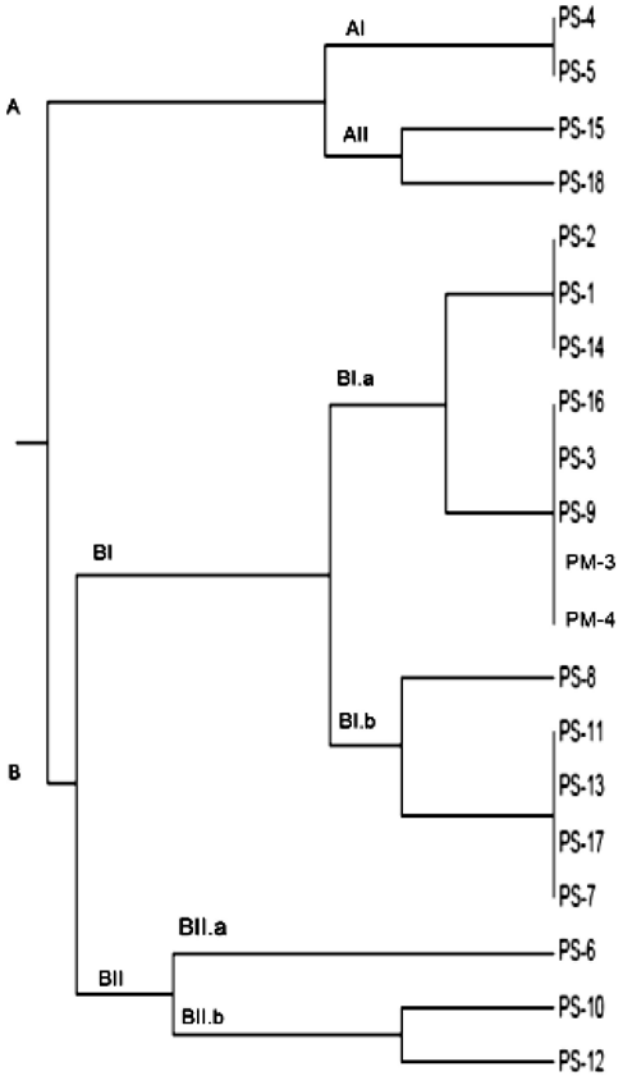

Fig. 2. Dendrogram of the pummelo genotypes based on the SSR markers using the UPGMA method.

DECLARATION

The authors declare no conflict of interest.

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