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Diversity, characterization and evaluation in Pummelo (*Citrus maxima* Merr.) cultivars using SSR markers and quality parameters

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Abstract

Fourteen pummelo (*Citrus maxima* Merr.) fruit varieties were evaluated through morphological and molecular methods to determine the genetic diversity among them. The analysis showed that maximum contribution (60%) towards diversity was due to the number of fruits per tree and rag percentage. Principal component analysis explained 80.26% of the total observed variability. Molecular characterization of pummelo varieties using 60 SSR markers revealed 26 polymorphic SSR loci having 77 amplified alleles and the number of alleles ranged from 1 to 4 with an average of 2.96 alleles per locus. The highest number of alleles per locus recorded was four as amplified by the SSR markers, CAT01, CS05, CCSM70, CIBE5156, AG14, CIBE4728 and CMS26. The PIC value ranged from 0.12 (CIBE5720) to 0.73 (CAT01) with average value of 0.53. Maximum heterozygosity was found in CAT01 (0.73) followed by CS05 (0.72) and AG14 (0.69). Pink Pummelo and White Pummelo showed the highest genetic similarity having coefficient of 89% and were closely related. The present study indicated low genetic diversity in pummelo varieties despite having high morphological variability, which could be elucidated by the fact that much of the phenotypic variation witnessed may be due to somatic mutations.

Key words: Pummelo, Characterization, SSR markers, physico-chemical parameters, assessment

Introduction

Citrus is grown commercially in tropical as well as sub-tropical regions of the world. The genus *Citrus* belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family (Webber 1967). Pummelo [*Citrus maxima* (Burn.) Merr. 2n=18] is known as one of the important commercial fruit tree under the genus *Citrus* (Verdi 1988). It is a native plant species to tropical and subtropical regions in Asia and has been cultivated in China for over 2000 years (Corazza-Nunes et al. 2002; Yong et al. 2006). It was originated from South East Asia, and named as shaddock in the western regions (Uzun and Yesiloglu, 2012). *C. maxima* is one of the three true *Citrus* species together with *C. medica* and *C. reticulata* (Barrett and Rhodes 1976; Hynniewta et al. 2011). Its status as true or basic species with in citrus is also confirmed by other researchers (Barkley et al. 2006; Froelicher et al. 2011; Garcia-Loret et al. 2013). Therefore, pummelo has been regarded as a parent of many citrus fruits, such as lemons, oranges and grapefruits.

Pummelo fruits are highly monoembryonic unlike other citrus species with distinguished features of huge leaves borne on broadly winged petioles, very large and fragrance flowers and big fruits with a single embryo (Uzun and Yesiloglu 2012). In India, citrus is grown in home gardens in all states of India and the maximum diversity is reported from North-East (NE) region (Singh and Singh 2003; Roy et al. 2014), Bihar and Bengal. Genetic variability in citrus is believed to be the result of many factors, such as mutation, hybridization and type of reproduction (mostly apomictic). Genetic diversity analysis studies in citrus have been reported by many researchers (Barkley et al. 2006; Jannati et al. 2009; Biswas et al. 2010; Garcia-Lor et al. 2012), and in particular, lemons (Gulsen and Roose, 2001) sweet orange (Novelli et al. 2006), as well as grapefruit (Corazza-Nunes et al. 2002) and pummelo (Barkley et al. 2006; Liu et al. 2006). These studies concluded that pummelo had greater genetic diversity as compared to other species.

Morphological study is very important component for the assessment of diversity and

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classification. Pummelo is also an important gene pool for breeding new types of citrus, and a dozen of pummelo-derived cultivars have been released in the past years such as grapefruits, tangelos, and hybrids (Nicolosi et al. 2000; Barkley et al. 2006; Garcia-Lor et al. 2012). According to Wen et al. (2010), there is an urgent need for pummelo conservation due to a decline in varietal diversity owing to loss of natural habitat, elimination of unwanted phenotypic characters and artificial selection during domestication process. Pummelo has tendency of declining varietal diversity due to loss of natural habitat so there is an urgent need of its conservation (Wen et al. (2010) and therefore, the problem of decreasing variability needs an utmost attention to prevent further loss of plant species that has not been fully uncovered. The variability available in pummel germplasm has been used by breeders to practically distinguish different cultivars and further for crop improvement. Keeping in view the versatility of morphological characters and molecular marker in characterization of germplasm, and the availability of wide range of pummelo germplasm at Punjab Agricultural University, Ludhiana, the present study was conducted to analyse the diversity in pummelo germplasm.

Materials and methods

The study was carried out on fourteen pummelo cultivars namely, CHS-Pink, CHS-White, Devanpalli, NRCC Pummelo-1, NRCC Pummelo-2, NRCC Pummelo-3, NRCC Pummelo-4, NRCC Pummelo-5, PTF-1, PTF-2, PTF-3, PTF-4, White Pummelo and Pink Pummelo (Table 1) in the Department of Fruit Science, Punjab Agricultural University, Ludhiana, India during the years 2015-17. Observations were recorded for different morphological characters at different growth and development stages. All the trees received recommended doses of fertilizers and other cultural practices during the course of these investigations.

Morphological evaluation

Evaluation for morphological parameters was conducted for two years i.e., 2015 and 2017 and analysed from pooled data. Quality parameters were recorded on matured fruits harvested at appropriate stage. Characterization was done on the basis of International Plant Genetic Resources Institute (IPGRI) citrus descriptors (IPGRI 1999). Twenty randomly selected fruits in each genotype were used for recording morphological traits and quality parameters *viz*., number of seeds per fruit, peel thickness, fruit weight, fruit size, juice content (%), peel content (%) and rag content (%). Total soluble solids (TSS) were determined by using digital refractometer and total titratable acidity (% of citric acid) was determined using N/10 NaOH and phenolphthalein as indicator. Ascorbic acid (mg/100 ml of juice) was estimated by using dye (2, 6-dichlorophenol indophenol) according to the method proposed by Rangana (1986). The experiment was conducted in randomized block design with five replications. Data were subjected for analysis of variation to one way ANOVA. Statistical analysis was performed using analysis of variance. P values d" 0.05 were considered as significant. A cluster analysis was performed using the unwieghted pair group method with arithmetic average (UPGMA) based on simple matching coefficient in NTSYS software. As per formula described by Burton (1952) and Burton and de Vane (1953), phenotypic and genotypic coefficients of variation were calculated. Broad sense heritability was calculated as suggested by Allard 1960) and genetic advance per cent of mean was calculated as per the method suggested by Johnson et al. (1955).

Isolation and purification of genomic DNA and selection of primers

Genomic DNA was extracted from young leaves (3 weeks old) of five randomly selected plants in each accession and then bulked for subsequent analysis as per the prescribed protocol (Gusmini et al. 2004). In 50 l of 1X TE buffer (Tris-EDTA buffer-10 mM Tris-HCl, I mM EDTA, air dried DNA pellets were dissolved and pH 8.0 is maintained. Quality and quantity of DNA was determined by NanoDrop 1000 instrument (Thermo Scientific, USA) using 2 µl of genomic DNA. Absorbance was recorded at 260/280 nm and readings were taken for both the quantity (ng µ/l) and quality (absorbance). Only the samples with absorbance value from 1.90-2.00 were taken for DNA analysis. 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. PCR amplification of 20 µl total volume was performed in 2.0 µl of 10X PCR buffer, 2.5 µl 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.

Data collection and analysis

The phenotypic diversity among pummelo varieties was computed on the basis of quantitative morphological characters by using 9.3 version SAS (Statistic analysis software).

SSR alleles were scored for the absence (0) and presence (1) of the SSR bands. Polymorphism information content (PIC) for each SSR marker was determined as per the procedure outlined by (Senior et al. 1998).

$$\Sigma (Pij)^2$$

i=1 Where; *Pij* is the frequency of j^{th} allele in ith 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 hierarchical classification (Rohlf 1989) by employing Unwieghted Pair Group Method using Arithmetic Averages (UPGMA). For estimating the similarity matrix, null alleles (no SSR allele 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).

$$GD = n\,(1-p^2)/(n-1)$$

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

Results and discussion

Morphological diversity and cluster analysis

The results on morphological characterization are presented in Tables 1 and 2. Based on the characterization, variety Devanpalli was classified as tall with maximum height and produced higher number of seeds/fruit, whereas CHS-Pink and CHS White produced lowest no. of seeds. Also the leaf area (cm^2) was maximum in Devanpalli followed by NRCC Pummelo-2. Devanpalli also showed high pollen viability and maximum pollen germination (%) while CHS Pink recorded minimum value for both the traits. Basabal et al. (2017) also observed higher values of pollen germination in a few genotypes and high pollen viability in NRCC Pummelo-2 indicating that the result of present findings are well supported by previous studies. The fruit weight (g) of PTF-3 was higher (1468.5) while minimum fruit weight was recorded in CHS White (975.2). Morphological characterization of pummelo has been studied by several researchers (Uzun et al. 2010) who found that the variation of fruit weight was more or less similar to those studied by Ara et al. (2008) and Samarasinghe (2005). However, Mitra et al. (2011) obtained more wide range of fruit weight (570-2010 g). Fruit diameter and fruit length (mm) were also recorded highest in Devanpalli whereas CHS White recorded the lowest values for these traits. The TSS (°Brix) value was higher in White Pummelo as compared to CHS Pink (7.2), which was lowest. In case of ascorbic acid (mg/100g) NRCC Pummelo-2 showed maximum value (46.2) and the White Pummelo (32.5) showed minimum (Table 1); juice content (%) was recorded maximum in Devanpalli (17.4) while rag content (%) was maximum in PTF-3 (66.2). Similarly, Baswal et al. (2017) while working on morphological characters of six pummelo genotypes concluded that maximum viable pollen was obtained from the anthers of NRCC Pummelo 2 (79%) followed by NRCC Pummelo 3 (74.75%) while the minimum viable pollens were obtained from the anthers of Pink Pummelo (46.75%). The results on mean fruit weight (741.30-1260.0 g/fruit), mean total soluble solids (7.57-8.44 brix) and mean titrable acidity (0.77-1.02%) were comparable.

Cluster analysis divided pummelo varieties into four groups (Fig. 1). Clusters I, III and IV consisted each of three varieties, whereas cluster II comprised of five varieties. Cluster I having CHS Pink, CHS White and Devanpalli, in which CHS Pink and CHS White were closely associated, whereas Devanpalli having

Table 1. Morphologicaland physio-chemical parameters of pummelo verities (pooled data for two years)

Varieties	Fruit diameter (mm)	Fruit length (mm)	Fruit rind thickness (mm)	No. of segments/ fruit	Total soluble solids (°Brix)	Acidity (%)	pH	Ascorbic acid (mg/ 100g)	Juice content (%)	Peel content (%)	Rag content (%)	Fruit weight (g)
CHS Pink	137.6	119.1	13.8	14.4	7.2	1.21	3.3	43.6	9.4	43.7	46.7	1021.5
CHS White	122.9	125.0	14.9	15.0	7.4	1.43	4.1	45.6	13.8	44.7	41.3	975.2
Devanpalli	196.8	179.2	14.3	18.4	8.6	0.87	3.3	32.9	17.4	51.3	31.3	1547.0
NRCC Pummelo-1	163.0	131.9	17.9	15.1	8.3	0.93	3.9	34.7	14.4	49.5	36.3	1241.0
NRCC Pummelo-2	189.9	172.9	16.4	15.7	7.6	1.06	4.2	46.2	13.8	47.1	39.3	1349.3
NRCC Pummelo-3	181.8	162.1	16.9	19.8	9.2	0.82	3.7	41.5	13.9	48.0	38.2	1284.7
NRCC Pummelo-4	186.9	168.9	15.3	16.4	8.8	0.69	3.4	39.5	14.8	46.2	38.9	1297.0
NRCC Pummelo-5	167.5	144.8	16.0	17.3	8.3	0.94	4.1	41.1	15.8	49.9	39.3	1302.2
Pink Pummelo	149.5	136.9	21.7	18.6	9.5	0.77	4.4	33.5	11.1	63.2	25.7	1187.6
PTF-1	172.2	147.5	20.3	13.8	8.0	1.00	4.9	37.4	13.3	53.7	32.9	1368.8
PTF-2	176.1	160.7	21.0	13.3	9.8	0.83	3.5	38.3	12.9	56.9	30.2	1421.9
PTF-3	177.8	157.6	28.6	14.5	9.3	0.77	3.6	42.4	12.5	66.2	21.3	1468.5
PTF-4	142.9	151.3	22.7	15.5	7.9	1.12	4.8	35.8	13.4	58.5	28.3	1048.4
White Pummelo	155.3	142.6	24.0	16.4	10.0	0.67	4.0	32.5	14.5	61.6	23.9	1124.9
LSD (p=0.05)	6.9	5.5	1.6	1.5	0.8	0.07	0.3	2.6	1.3	4.2	2.7	44.7
SEm±	2.4	1.9	0.5	0.5	0.3	0.02	0.1	0.9	0.5	1.4	0.9	15.3

0.24 cm distance from other varieties was poorly associated with other varieties. In cluster II (NRCC Pummelo-1, NRCC Pummelo-4, White Pummelo, NRCC Pummelo-3 and NRCC Pummelo-5), NRCC Pummelo-1 and NRCC Pummelo-4 were closely associated with an average distance of 0.002 cm while, NRCC Pummelo-3 and NRCC Pummelo-5 were less closely associated with each other with an average distance of 0.09 cm. Cluster III consisted of three varieties viz., NRCC Pummelo-2, Pink Pummelo and PTF-4, whereas PTF-4 and Pink Pummelo were closely associated with an average distance of 0.008 cm and NRCC Pummelo-2 had 0.14 cm distance from other varieties with poor association. Likewise cluster IV consisted of varieties namely, PTF-1, PTF-2 and PTF-3. PTF-2 and PTF-3 were closely associated with an average distance of 0.08 cm while, PTF-1 was less associated with other varieties with an average distance of 0.22 cm. The varieties were clustered irrespective of their geographical area of collection. The morphological variation in pummel varieties under field conditions could be due to the action of evolutional forces and environmental attributes (Paudyal and Haq 2008). However, Dorji and Yapwattanaphun (2011a) hypothesised that morphological variation among the accession could be attributed to mutations, cross pollination and genotype x environment interactions. Various research workers have reported different groups with varied degree of dissimilarity studying different sets of varieties of mandarin (Koehler-Santos et al. 2003; Singh et al. 2016), grapefruit (Sharma et al. 2015), citrus (Abedinpour et al. 2015; Marboh et al. 2015). The SSR and RAPD markers grouped the citrus genotypes of rough lemon into clusters (Sanabam et al. 2018), an acidic citrus group of lemon or natural hybrids and a small cluster of three genotypes where the genotypes in other clusters were having genetic affinity as well as some were distinct. The findings of the present study are supported that pummelo genotypes were clustered in different groups irrespective of their geographical area of origin. Baswal et al. (2017) subjected the morpho-physiological data to cluster analysis and grouped the pummelo genotypes into four clusters indicating dissimilarity for morphological features such as fruit weight, fruit diameter, fruit length, fruit rind thickness and number of segments per fruit and performance with respect to total soluble solids and 20 seeds weight among the genotypes. A higher range of variation with average Euclidean distance coefficient ranging from 0.09-3.77 was observed between groups as well as clusters, thereby indicating that genotypes within and among

Table 2. Morphological parameters of pummelo verities (pooled data for two years)

Varieties	Av. no. of seeds/ fruit	Seed weight/ fruit (g)	Root-stock dia-meter	Scion dia-meter	Spine dia-meter	Leaf lamina length (mm)	Leaf lamina width (mm)	Leaf area (cm^2)	Petiole wing width (mm)	Flower diameter (mm)	Pollen viability (%)	Pollen germi-nation (%)
CHS Pink	68.2	39.6	91.6	89.1	3.8	120.7	68.9	41.6	8.9	38.7	53.7	40.8
CHS White	74.5	42.5	92.4	87.3	4.7	123.4	71.3	45.2	9.3	43.4	56.7	43.5
Devanpalli	156.7	52.8	102.0	100.4	4.6	138.6	85.3	57.7	27.4	66.6	74.3	60.2
NRCC Pummelo-1	121.4	41.6	95.1	92.2	4.9	121.8	65.7	43.5	19.3	58.8	72.0	58.4
NRCC Pummelo-2	118.4	50.8	100.9	100.5	5.2	134.1	81.6	53.5	18.4	60.6	71.4	57.6
NRCC Pummelo-3	142.7	46.6	102.6	99.4	3.8	126.8	76.7	48.9	21.1	57.4	62.5	51.7
NRCC Pummelo-4	92.5	48.8	88.8	87.0	4.3	131.2	80.7	50.8	21.9	58.0	60.2	49.6
NRCC Pummelo-5	98.6	43.5	90.1	88.2	2.3	124.7	73.5	42.9	20.1	56.5	59.2	47.8
Pink Pummelo	102.5	28.6	87.3	86.6	4.1	99.6	53.5	28.8	12.8	49.3	60.2	50.0
PTF-1	119.8	31.3	97.3	92.8	3.9	114.4	68.9	36.5	16.8	46.6	69.3	54.4
PTF-2	122.6	21.8	92.1	89.7	3.5	101.3	56.7	30.7	15.9	48.6	63.4	51.6
PTF-3	128.5	23.3	93.7	91.3	3.3	103.3	54.2	32.6	19.8	53.6	66.7	53.2
PTF-4	137.0	25.6	93.1	85.0	3.8	110.2	60.7	34.6	23.8	61.8	68.3	58.5
White Pummelo	132.5	26.8	89.8	86.8	5.2	98.7	52.7	26.8	11.8	51.8	65.9	45.2
LSD (p=0.05)	7.2	2.7	4.6	4.0	0.5	6.0	5.2	4.9	1.6	3.7	4.1	4.0
SEm±	2.5	0.9	1.9	1.7	0.2	2.1	1.8	1.7	0.5	1.3	1.4	1.4

clusters were highly diverse. Knowledge of such range of genetic diversity among the genotypes will facilitate its use in management and conservation and in selection of cross-parents in breeding programs.

Principal coordinates analysis

The principal coordinate analysis was performed for better visualization of relationship among the morphological and quality traits in pummel varieties (Tables 1, 2 and Fig. 2). Principal component analysis (PCA) was used considering the value of 34 morphological (roots, leaves, fruit and floral traits) and quality traits to identify the most significant variables in the data set produced from three principal components. These components explain 80.26% of the total observed variability. Value of the extracted information is adequate considering the number of involved variables and the study's purpose. The first component presented 46.01% of the variation and variables with higher scores on PC1 (over 0.21, absolute value) related to pollen germination, fruit length, diameter of fruit axis and tree trunk circumference were positively correlated while the characters like pH, TSS and acid ratio with higher scores are negatively correlated. The second component explained 25.07% of the total variation and features like leaf lamina length, leaf lamina width and leaf area were positively correlated while the TSS, fruit rind thickness and peel percentage are negatively correlated. The third component accounted for 09.18% of the variation in which root stock circumference, scion trunk circumference and ratio scion to rootstock circumference are positively correlated while the characters like flower diameter, staminate percent flowers and leaf lamina length to width ratio are higher with negatively correlated. In PC1, which explained the largest proportion of variability, as many as 6 traits showed a high loading. The summarization of these traits in one component reflected the strong correlation between scion circumference, rootstock circumference and ratio between scion and rootstock circumference and fruit length fruit diameter and pollen germination**.** Similarly, in PC2, three traits, leaf lamina length, leaf lamina width and leaf area that had the most considerable loading were significantly correlated with each other. The result suggested a reduction of these traits to three main characters, namely, rootstock and scion, leaf and fruit could be sufficient. Highest variability was shown by Devanpalli, followed by NRCC Pummelo-2 and NRCC Pummelo-3 and least variability

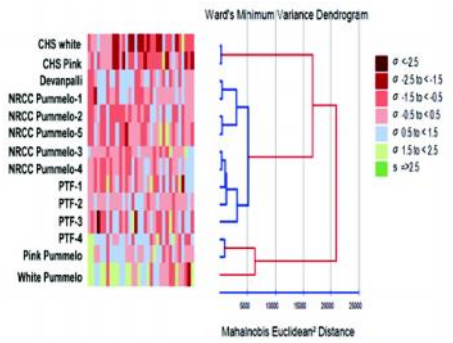

Fig. 1. Overall proportion of pummelo admixture estimated from diagnostic polymorphisms for different varieties

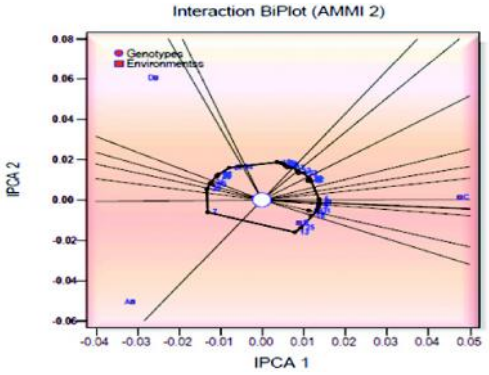

Fig. 2. Dispersion of different pummelo on the two-dimensional plot of the factorial correspondence analysis

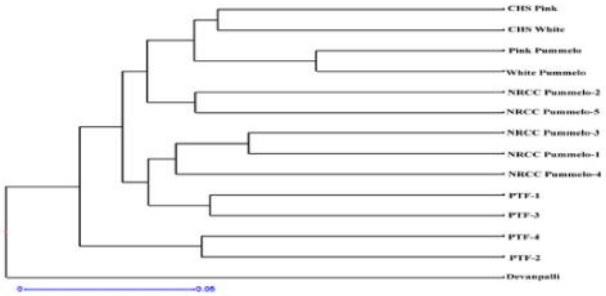

Fig. 3. Dendrogram illustrating genetic relationship among different pummelo varieties generated by UPGMA tree analysis

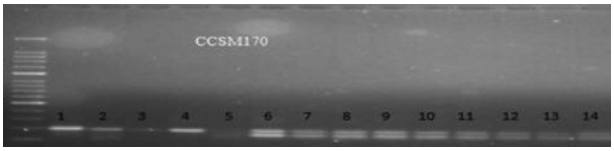

Fig. 4. Agarose gel showing SSR amplification profile by different primer in different pummelo varieties. 1. CHS-Pink, 2.CHS-White, 3.Devanpalli, 4.NRCC Pummelo-1, 5.NRCC Pummelo-2, 6. NRCC Pummelo-3, 7. NRCC Pummelo-4, 8.NRCC Pummelo-5, 9.Pink Pummelo, 10. PTF-1, 11.PTF-2, 12.PTF-3, 13.PTF-4 and 14.White Pummelo

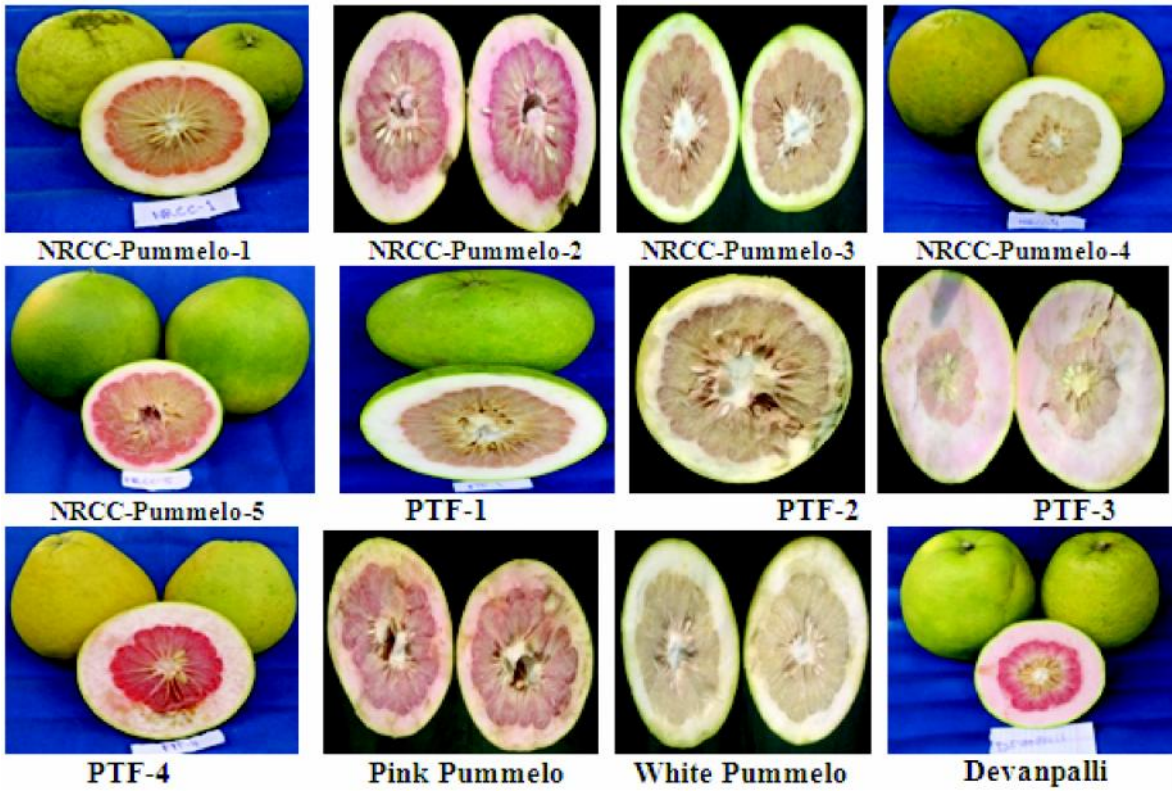

Fig. 5. Genetic diversity in fruit size and shape of different pummelo varieties

Table 3. Variability, heritability and genetic advance

Characters (Pummelo)	PV	GV	PCV	GCV	h^2(%)	GA (% of mean)
Leaf lamina length (mm)	186.6	173.9	11.6	11.2	93.1	22.2
Leaf lamina width (mm)	128.4	119.0	16.7	16.0	92.7	31.8
Rootstock bud circumference (cm)	26.3	24.0	15.3	14.6	91.2	28.9
Leaf area	98.0	90.4	24.2	23.1	91.5	45.7
Scion truck circumference	26.5	25.1	16.8	16.4	94.7	32.
Average number of seeds per fruit	655.7	637.4	22.1	21.8	97.2	44.4
Seed weight per fruit (g)	119.4	116.9	29.2	28.9	97.9	58.9
Seed length (mm)	1.6	1.3	7.8	7.1	81.8	13.2
Seed width (mm)	0.7	0.6	12.1	10.8	79.5	19.8
Flower diameter (mm)	62.9	58.	14.7	14.2	92.2	28.4
Staminate (%)	2.0	2.0	30.7	30.2	97.1	61.4
Perfect (%)	2.4	1.6	1.6	1.3	67.5	2.32
Pollen viability (%)	41.8	35.8	10.0	9.2	85.6	17.6
Pollen germination	38.8	33.2	12.0	11.1	85.5	21.2
Fruit diameter (mm)	489.3	472.6	13.3	13.1	96.6	26.5
Fruit length (mm)	328.6	317.8	12.0	11.8	96.7	24.0
Fruit rind thickness (mm)	19.4	18.5	23.4	22.8	95.4	46.0
Number of segments per fruit	4.1	3.3	12.7	11.5	81.3	21.3
Diameter of fruit axis (mm)	9.2	7.5	14.0	12.6	81.0	23.3
Total soluble solids (%)	0.9	0.7	11.3	9.9	76.6	17.9
Acidity (%)	0.0	0.0	23.1	22.7	96.3	45.9
Ph	0.2	0.2	13.6	13.0	91.8	25.7
VIT C	23.1	20.7	12.3	11.7	89.6	22.8
JUICE %	4.1	3.5	14.9	13.8	84.9	26.1
PEEL %	56.8	50.6	14.2	13.4	89.0	26.1
RAG %	55.8	53.3	22.1	21.6	95.4	43.5
Number fruits per tree	676.5	667.1	30.5	30.3	98.6	61.9
Fruit weight (g)	29869.8	29167.5	13.7	13.5	97.6	27.6

Where PV= phenotypic variance, GV= genotypic variance, PCV= phenotypic coefficient of variance, GCV= genotypic coefficient of variance, h^2= heritability in broad sense and GA= genetic advances

was observed in CHS Pink (Table 3). High absolute values of the correlations between variables related to fruit and leaf size, and PC1 or PC2 were also established earlier (Krahl et al. 1991; Rakonjac et al. 2010) in sour cherry and other characters of fruits and leaves in papaya (Asudi et al. 2010) and *Pyrus pyraster* **(**Paganova 2009). Further, Shrestha et al. (2012) demonstrated the role of morphological characters in distinguishing five landraces of *Citrus aurantifolia*, whereas Martasari and Reflinur (2012) in Indonesian *Citrus nobilis* and subsequently used those characters as the main basis in genotype selection for breeding programs. This indicated that these traits could be sufficient for reliable germplasm characterization.

Contribution towards diversity revealed that the highest diversity (30%) was found in rag per cent and number of fruits per tree which was followed by productivity (13 %), fruit rind thickness (9%), pH (7%), Vit. C (3%) and peel per cent (3%) (Fig. 3). It was concluded that maximum contribution of about 60% towards diversity was due to number of fruits per tree and rag percentage. Marboh et al. (2015) concluded that high diversity observed within the genotypes of

Table 4. Number of alleles amplified, polymorphism (%), polymorphic information content (PIC) value and genetic diversity of SSR markers

S. No.	SSR marker	Polymorphic allele	Total no. of allele	Polymorphism (%)	PIC	Hetero zygosity	Genetic diversity
1	CS05	3	4	75	0.72	0.722	0.78
2	CCSME15	2	2	100	0.44	0.444	0.48
3	OP571	2	3	66.66	0.49	0.490	0.53
4	CAT01	4	4	100	0.73	0.735	0.79
5	CCSM77	3	3	100	0.60	0.595	0.64
6	CCSM156	2	3	66.66	0.64	0.641	0.69
7	CCSM170	2	2	100	0.48	0.480	0.52
8	CCSM201	3	3	100	0.66	0.561	0.71
9	CCSM204	3	3	100	0.53	0.531	0.57
10	CCSM68	2	2	100	0.36	0.360	0.39
11	CCSM70	3	4	75	0.52	0.517	0.56
12	CCSME15	2	2	100	0.24	0.320	0.26
13	CIBE5156	3	4	75	0.61	0.614	0.66
14	CL11	2	3	66.66	0.53	0.533	0.57
15	CS06	1	2	50	0.42	0.420	0.45
16	CS09	3	3	100	0.63	0.635	0.68
17	CIBE5720	1	2	50	0.12	0.124	0.13
18	GT03	2	3	66.66	0.41	0.410	0.44
19	AG14	3	4	75	0.70	0.697	0.75
20	0P29	3	3	100	0.55	0.553	0.60
21	CMS46	1	2	50	0.49	0.486	0.52
22	CMS09	2	3	66.66	0.55	0.552	0.59
23	CIBE4728	3	4	75	0.63	0.631	0.68
24	CMS26	2	4	50	0.71	0.604	0.77
25	CMS30	2	2	100	0.47	0.465	0.50
26	AG14	3	3	100	0.56	0.561	0.60
	Total	62	77	2108.3	13.79	11.720	14.86
	Mean	2.38	2.96	81.09	0.53	0.451	0.57

mandarin was comparable to sweet orange and grapefruit, which points to ample possibilities of obtaining desirable trait combinations in specific cultivars. The traits shows high diversity in traits may take in consideration for further varietal improvement programme in citrus. Both quality and yield traits should be observed for diversity so as to meet the demand of consumer's choice of present era.

Variability, heritability and genetic advance in pummelo varieties

Estimation of phenotypic variance (PV) and genotypic variance (GV) among the genotypes indicated that maximum variance was recorded for fruit weight, productivity, number of fruits per tree and number of seeds per fruit (Table 3). The variation present in population is due to genotypic and environmental and their interaction effects. The highest phenotypic coefficient of variance (PCV) and genotypic coefficient of variance (GCV) were recorded for number of fruit per tree, seed weight, fruit rind thickness and number of seeds per fruit (Table 3). All these above mentioned characters were highly influenced by the environment as compared to other characters. In the present investigation, genetic advance coupled with high heritability was also observed for productivity, seed weight per fruit, fruit rind thickness, acidity, no. of seeds per fruit and no. of fruits per tree. Recently, Ahmed et

al. 2018) concluded that in case of grapefruit, the highest PCV and GCV were recorded for seed weight, no. of seeds per fruit, no. of fruits per tree and acidity. These characters were also influenced by the environmental factors. Earlier, Panse (1957) had suggested that if heritability is mainly due to additive genetic effects, a high genetic advance coupled with high heritability may be expected. Under these conditions, it is expected that the selection would be highly effective for seed weight, no. of seeds/fruit, no. of fruits/tree and acidity.

Molecular characterization

Sixty SSR primer pairs belonging to diverse series were used for identification and evaluation of genetic diversity in 14 pummelo varieties (Table 4). The primer pairs, CCSM06 and AC01 failed to show any amplification thus revealing no band (null allele) in all the varieties of pummelo. Of the 57 SSR markers amplified, 26 exhibited polymorphism in pummel varieties and showed high level of allelic diversity while remaining were monomorphic. Allelic frequency or the frequency at which alleles are found at any locus of interest is used to estimate the frequency of given genetic profile. Among pummelo varieties a total of 77 alleles were amplified by 26 polymorphic SSR loci and the number of alleles ranged from 1 to 4 with an average of 2.96 alleles per locus (Table 4). The highest number of alleles per locus was four as amplified by CAT01 CS05, CCSM70, CIBE5156, AG14, CIBE4728 and CMS26 followed by three alleles per locus each by and the remaining markers amplified two alleles. The results of present study are in agreement with earlier findings (Barkley et al. 2006; Jannati et al. 2009) which reported different SSR primers and concluded that CAT01 is highly informative marker in citrus and we even find its effectiveness in case of pummelo species too.

Genetic diversity among pummelo varieties ranged from 0.13 (CIBE5720) to 0.79 (CAT01) and the average value across all the primers was 0.57 (Table 4). The analysis of genetic relationship classified the genotypes in to major 3 clusters major clusters (I, II and III). The cluster I contained Devanpalli, PTF-2 and PTF-4 variety but cluster II was further sub divided into two sub clusters IIIA with two varieties, PTF-3 and PTF-1, while III B had three varieties, NRCC Pummelo-4, NRCC Pummelo-1 and NRCC Pummelo-3. Cluster III was further sub divided into two clusters, IIIA with two varieties, NRCC Pummelo-5 and NRCC Pummelo-2 and cluster IIIB having four varieties, White Pummelo, Pink Pummelo, CHS Pink and CHS White. In present study pummelo genotypes had however, low level of genetic diversity despite having high morphological variability suggesting that much of phenotypic variation may be because of somatic mutations. Similar findings were reported in grapefruit (Novelli et al. 2000; Bretó et al. 2001; Yong et al. 2006), sweet orange (Malik et al. 2012) and mandarin genotypes (Singh et al. 2016). Maximum heterozygosity among the genotypes was reflected by the marker, CAT01 (0.73) followed by CS05 (0.72), AG14 (0.69) (Table 4). Similarly, Barkley et al. (2006) reported high level of heterozygosity among certain citrons e.g., Citron of Commerce had 52.17% heterozygous SSR markers, Italian citron had 43.38% heterozygosity and one known citron hybrid (CRC3819) had a high percentage of heterozygosity at 82.61%. The varieties Pink Pummelo and White Pummelo showed the highest genetic similarity having coefficient of 0.89 and were closely related. However, Devanpalli and NRCC Pummelo-4 showed the lowest (0.63) genetic similarity coefficient and these were genetically distinct from each other. In a few studies conducted by Corazza-Nunes et al. (2002) in grapefruit and by Gulsen and Roose (2001) in lemons (*C. limon*) using isozymes, SSR and ISSR markers also recorded a similarity level of genetic similarity ranging from 0.98 to 1.00, which are in good agreement. Sanabam et al. (2018) using SSR and RAPD molecular markers, characterised 18 citrus genotypes of rough lemon strains and other under-utilized *Citrus* spp. collected from North-East region of India unraveled the genetic relationship among the rough lemons. The study will be of immense value for conservation and utilization of the region's rich citrus resources.

In case of pummelo varieties, the percentage of polymorphism of the 26 polymorphic markers ranged from 50 to 100. Among these, 11 exhibited 100% polymorphism; five having 75%, another five showed 66.66% and remaining have 50% with an average polymorphism (%) was 81.09. The PIC value which is a measure of allelic diversity at a locus ranged from 0.12 (CIBE5720) to 0.73 (CAT01) with an average value of 0.53. Fifteen SSR markers revealed PIC value more than 0.52. Primer CAT01 amplified 4 alleles and had a highest PIC value of 0.73 followed by CS05 in which 3 alleles was amplified and had PIC value of 0.72 (Table 6). All the alleles amplified by CAT01 primer pairs on all the varieties of pummelo were all distinguishable. It has been observed that marker GT03 amplified 3 alleles and had PIC value of 0.41 while CSM46

amplified 2 alleles and had PIC value of 0.49. Therefore, there seemed to be no strong correlation between the PIC value and the number of alleles amplified. Across all varieties, a total of 873 alleles were amplified by 58 SSR primers with an average of 62.36 alleles for each variety. The average amplified fragments for polymorphic marker was 46.00 whereas for monomorphic, it was 16.36. The maximum number of alleles (68) was detected in NRCC Pummelo-3 where as PTF-2 showed least number (51) alleles. However, the per cent of polymorphic markers was maximum (80.60) in White Pummelo followed by (79.69) per cent in NRCC Pummelo-2. The present findings are supported by Meral et al. (2011) in Satsuma mandarins among them a narrow genetic diversity in clones was recorded. They further reported that the observed morphological polymorphism within Satsuma mandarins must be associated with somatic mutations which were not detected by SSR molecular markers. Similar observations were recorded by Singh et al. (2016) in 19 different mandarin genotypes with maximum 5 alleles amplified with an average of 2.46 alleles per primer pair. The highly informative marker CAT01 produced maximum number of alleles. On the basis of diversity analysis of pummelo genotypes, it could be concluded that the pummelo genotypes can be successfully used for planning future breeding programmes to obtain hybrids with desired traits. Combination with high heterotic response and superior recombinants may be obtained through hybridization between genotypes across the clusters.

Authors' contribution

Conceptualization of research (SA, HSR); Designing of the experiments (SA, HSR, GS); Contribution of experimental materials (SA, HSR, GS); Execution of field/lab experiments and data collection (SA); Analysis of data and interpretation (SA, HSR, GS); Preparation of the manuscript (SA).

Declaration

The authors declare no conflict of interest.

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