DIVERSITY ASSESSMENT OF GRAPEFRUIT (*CITRUS × PARADISI*) AND TANGELO (*CITRUS × TANGELO)* UNDER INDIAN CONDITIONS USING PHYSICO-CHEMICAL PARAMETERS AND SSR MARKERS

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Abstract. In a previous report, grapefruit (*Citrus × paradisi* Macfad.) and tangelo (*Citrus × tangelo* J.W. Ingram & H.E. Moore, 1975) landraces from diversity rich Indo-Gangetic Plain and arid regions of India were characterised using specific SSR markers. Eight grapefruit and two tangelo varieties were compared with germplasm of diverse origin comprising of cultivars, landraces and wild types. Significant differences were observed for different morphological characters studied. The highest phenotypic co-efficient of variance (PCV) and genotypic co-efficient of variance (GCV) were determined for seed weight (104.4 and 103.7, respectively), number of seeds per fruit (102.3 and 101.9, respectively), number of fruits per tree (60.0 and 60.0, respectively) and acidity (40.4 and 38.8, respectively). These mentioned characters were more influenced by environment than other characters. A total of 75 alleles were amplified by 26 polymorphic simple sequence repeat (SSR) loci and the number of alleles ranged from 1 to 4 with an average of 2.88 alleles per locus. The highest number of alleles per locus was four as amplified by CAT01, CS05, CCSM204, CCSM70, CIBE5156, ATC09 and CMS26 followed by three alleles per locus each by CCSM77, CCSM156, CL11, OP29, CMS46, CMS09, CIBE4728, CMS3O and AG14 and the remaining markers amplified two alleles. The highest number of alleles among grapefruit varieties was found in Ray Ruby (75) while the lowest number of alleles was found in Flame (61) while among tangelo varities the highest number of alleles was found in Pearl (64). Average polymorphism (%) of all the polymorphic primer pairs across all the varieties of grapefruit was 69.55 and the highest polymorphism among grapefruit varieties was found in Marsh (82.09) while among tangelo varieties the highest polymorphism was found in Pearl (81.25). The measure of allelic diversity that is PIC value ranged from 0.17 (CS06) to 0.75 (CAT01) with an average value of 0.53.
Keywords: *citrus, germplasm, morphological characterization, molecular markers, polymorphism*

Introduction

Citrus is a sub-tropical fruit that belongs to the family of *Rutaceae.* It is grown commercially throughout India and is known worldwide of its characteristic flavour, attractive evergreen foliage and flower as well as the extraordinary fragrance, which are added aesthetic values (Rabha et al., 2013). In India, it is grown in acreage of 935 thousand hectares with the production of 11515 thousand MT and productivity of 12181 MT/ha respectively (NHB, 2016).

The grapefruit (*Citrus × paradisi* Macfad.) originated from Barbados in the Caribbean islands, is a natural hybrid between pummelo (*Citrus maxima* (Burm.) Merr.) and sweet orange (*C. sinensis* L. Osb). It was first named as *Citrus paradise* Macfad. by James Macfadyen in 1837 (Scora et al., 1982 and Scora, 1988). The tangelo (*Citrus × tangelo* J.W. Ingram & H.E. Moore, 1975) probably originated in southeastern Asia over 3,500 years ago. Tangelo is most likely a result of insect cross pollination of the mandarin orange and the pummelo (pummelo is the ancestor of the grapefruit).

Grapefruit is the fourth most important citrus fruit economically in the world. It is rich in various nutrients, phytochemicals, vitamin C and fiberpectin with pink and red hues that contain the beneficial antioxidants lycopene (Silver et al., 2011). There are many benefits of this fruit worth mentioning: (i) peel and seed extract of grapefruit have antifungal properties (Okunowo et al., 2013), (ii) it helps in lowering cholesterol (Platt, 2000) and also (iii) in animals cell studies, grapefruit powder, limonin and naringenin decrease growth and increase self-destruction of colon, mouth, skin, lung, breast and stomach cancer (Chidambara et al., 2011). Citrus varieties show more diversity in their morphological traits such as size and shape of canopy, color, type, number of seeds, fruit weight, fruit diameter, flower diameter, TSS, acidity etc. Generally in plants, the diversity at the phenotypic level is much larger than at genetic level, as selectively neutral molecular markers are used to evaluate the extent of genetic variation.

For future variety management and conservation purposes this adaptive genetic diversity represents important potential. Recently researchers (Amara et al., 2011; Ahmed et al., 2012; Jianfeng et al., 2012; Uzun and Yesiloglu, 2012), have observed the significant diversity among cultivated genotypes of Citrus genus in respect of physiological, morphological and agronomic traits but very little DNA variation has been detected using DNA-markers. Likewise, in other cases markers were used to determine genetic diversity, characterization and phylogenetic relationships among the Citrus and related genera (Gulsen and Roose, 2001; Shahsavar et al., 2007; Uzun et al., 2009; Marak and Laskar, 2010; Golein et al., 2011). Grapefruits are highly polyembryonic and have low genetic variation due to nucellar and mutation origin (Fang and Roose 1997 and Corazza-Nunes et al., 2002). Substantial genetic variation among citrus species and cultivars is due to frequent bud mutation, interspecific and intergeneric hybridization, apomixes and long history of cultivation. There is an urgent need for diversification of scion citrus cultivars for Punjab citriculture for reducing the high risks associated with the outbreaks of new and more severe diseases and pests. Secondly, there is more variation in characters in citrus than in other fruit crops (Reuther et al., 1967) and citrus is phenotypically the most heterogeneous group (Moore, 2001). In the present study ten varieties of grapefruit and tangelo were studied with the aim (a) to correlate the physico-chemical variation with the genetic diversity (b) to estimate genetic polymorphism and relationships among grapefruit and tangelo varieties based on SSR markers.

Materials and methods

Plant material

In this study we investigate eight grapefruit and two tangelo cultivars (*Table 1*) from the College Orchard, Department of Fruit Science, Punjab Agricultural University, Ludhiana, India in the year 2015-16. 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

Morphological evaluation was carried out for two years, i.e. from 2015 to 2016. Fruits were harvested at full maturity, yield and quality parameters data were recorded.

Characterization of grapefruit and tangelo cultivars was conducted on ten genotypes on the basis of IPGRI (International Plant Genetic Resources Institute) citrus descriptors (Anonymous, 1999). Twenty fruits were sampled from each cultivar for quality analysis, and characteristics evaluated including number of seed per fruit, peel thickness, fruit weight, fruit size, juice content, peel content and rag content. Juice was filtered through filter paper, thereafter, juice samples were examined to determine the following parameters; pH, total soluble solids (TSS), titratable acidity (% of citric acid) using N/10 NaOH and phenolphthalein as indicator, using digital refractometer, and ascorbic acid (mg/100 ml of juice) using a dye (2, 6-dichlorophenol indophenol) according to the standard method (Rangana, 1986). Design of experiment was in randomized block with five replications. Data were subject for analysis of variation to one way ANOVA. Statistical analysis was performed using analysis of variance. P values ≤ 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. The phenotypic and genotypic coefficients of variation were calculated as per formula described by Burton (1952) and Burton and de Vane (1953). Heritability, in broad sense, was calculated as suggested by Allard (1960) and genetic advance percent of mean was calculated following the method suggested by Johnson et al. (1955).

Table 1. *Citrus paradisi Macf. cultivars and variants used in the study of germplasm characterization*

S. no.	Varieties	Origin
		Grapefruit
1	Flame	Originated in Florida – budwood registration program, winter haven in 1988
2	Foster	Originated as a branch sport of a selection called 'Walters' in Florida, USA in 1907
3	Marsh	Originated as seedling trees on the property of a Mrs. Rushin near Lakeland, Florida, USA (1862)
4	Ray Ruby	Originated in Texas in 1985
5	Red Blush	Originated as sports–lower branches–growing out of 'Thompson' trees in USA in 1929
6	Rio Red	Originated in Texas in 1985
7	Ruby Red	Originated in Texas in 1988
8	Star Ruby	Originated as a lower branch mutation bearing red-blushed fruits, noticed on a 'Foster' tree at San Benito, Texas, USA in 1930
		Tangelo
9	Minneola	Produced at John Carpenter, USDCS, Indio CA in 1961
10	Pearl	Hybrid produced at UCR in 1945

Isolation and purification of genomic DNA

Genomic DNA was extracted from 5 g of well ground tissue using the protocol described by Gusmini et al. (2004). DNA was extracted from young leaves of five randomly selected plants for each variety and then subsequent molecular analysis. Air dried DNA pellets were dissolved in 50 l of 1X TE buffer (Tris-EDTA buffer- 10 mM Tris-HCl, I mM EDTA, pH 8.0. Quantity and quality of DNA was determined by

Nanodrop 1000 instrument (Thermo Scientific, USA) using 2 l 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 having absorbance value from 1.90-2.00 were taken for DNA analysis. And the samples with absorbent values less are repeated till the desired amount of DNA is achieved.

Selection of primers

The DNA was amplified through polymerase chain reaction (PCR) using 60 SSR primer pairs (synthesized by 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 1 mM 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 (15 ng) 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 30 s, primer annealing at 48-57 °C for 1 min and primer extension at 72 °C for 1 min. 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 100 V for 2–3 h in 0.5 × TBE buffer. Gels were visualized under UV light and photographed using photo documentation system (Alphaimager 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. Polymorphism information content (PIC) for each SSR marker was determined as per the procedure outlined by Senior et al. (1998).

$$\text{PIC} = 1 - \Sigma \, (Pij)^2$$

$$i = 1$$

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 hierarchical classification (Rohlf, 1989) by employing Unweighted 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

Clustering studies based on morphological diversity

Grapefruit and tangelo varieties consist of four clusters (*Fig. 1*), cluster I consists of only one variety Flame with an average distance of 0.37 cm (*Table 2*) from other varieties. Cluster II consists of two varieties namely Marsh and Red Blush, which are more associated with each other with an average distance of 0.10 cm. While in cluster III there were four varieties namely Foster, Ray Ruby, Star Ruby and Rio Red. Foster have an average distance of 0.13 cm as compared to Rio Red which has 0.24 cm distance from the other varieties. In cluster IV there were three verities Ruby Red, Minneola and Pearl. Minneola and Pearl were closely associated with each other with an average distance of 0.25 cm while Ruby Red have less association with other varieties with an average distance of 1.37 cm from other varieties. Further, the study on inheritance of agronomic traits of citrus reports them to be controlled by multiple genes which can be assessed only through morphological assessment (Liu and Deng, 2007).

Table 2. *Root mean-square distance between grapefruit and tangelo varieties within clusters based on morphological characteristics.*

Number of clusters	Clusters joined		Freq	Norm RMS distance (cm)
9	Ray Ruby	Star Ruby	2	0.0136
8	Marsh	Red Blush	2	0.1072
7	Foster	CL9	3	0.1349
6	CL7	Rio Red	4	0.2447
5	Minneola	Pearl	2	0.2573
4	Flame	CL8	3	0.3715
3	CL4	CL6	7	0.5285
2	Ruby Red	CL5	3	0.7395
1	CL3	CL2	10	1.3799

However, Dorji and Yapwattanaphun (2011) concluded that the groups diverged having similarity coefficient of 0.79 in contrast to 0.41. There is not much variation with respect to qualitative characters of the groups. Golein et al. (2005) reported that the analyzed accessions having high average similarity coefficient (0.91) indicated that all mandarins represented variations of single clones. Likewise, analysis of 43 morphological characters in the 22 cultivars of *C. sinensis* revealed that maximum similarity (0.64) occurred between the cultivars Campbell Valencia and Vanale, both of which are exotic in origin and are most similar in terms of qualitative fruit, leaf and seed characters (Malik et al., 2012). Minimum similarity (0.18) was observed between the

cultivars Washington navel and Mosambi, which may be attributed to their different centres of origin where they have developed their distinct characters.
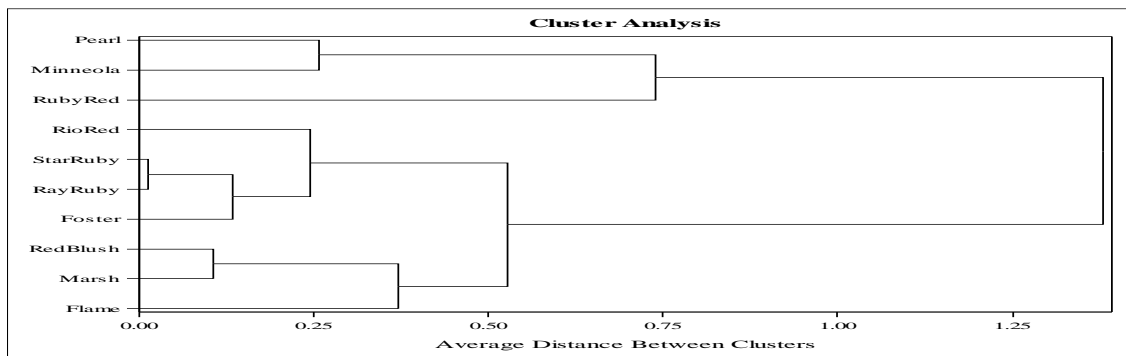

Figure 1. Dendrogram of grapefruit and tangelo varieties based on morphological characteristics using the UPGMA method

Variability, heritability and genetic advance in grapefruit and tangelo varieties based on morphological characteristics.

Estimation of genotype for phenotypic variance (PV) and genotypic variance (GV) indicated that variance was recorded maximum for number of fruits per tree, fruit weight, and fruit diameter, number of seeds per fruit and seed weight (*Table 3*). The variation present in population is due to genotypic and environmental effects. It includes both genotypic and environmental condition. The highest phenotypic co-efficient variance (PCV) and genotypic co-efficient variance (GCV) were recorded for seed weight (104.4 and 103.7, respectively), number of seeds per fruit (102.3 and 101.9, respectively), number of fruits per tree (60.0 and 60.0, respectively) and acidity (40.4 and 38.8, respectively). These mentioned characters were much more influenced by the environment than other characters.

In the present investigation of genetic advance coupled with high heritability was observed for seed weight, number of seeds per fruit, number of fruits per tree and acidity. Thus the results indicated that selection would be highly effective for above mentioned characters. Similar finding was made by Baswal et al. (2016), who concluded that the co-efficient of variation (both genotypic and phenotypic) was higher for the characters like density of oil glands/cm^2 (35.1 and 34.8, respectively) followed by albedo thickness (19.5 and 19.4, respectively). GCV associated with high heritability (80% or more) indicated that selection would be effective for the improvement of these characters. Also Roy et al. (2014) concluded that in pummelo germplasm while studying variability, a wide range of variability was observed for almost all variables or characters. The co-efficient of variation was higher (>20) for characters like yield per plant, fruit rind thickness, seed weight and number of fruits per plant.

Burton (1952) observed that if GCV associated with high heritability (80% or more) indicated that selection would be effective for the improvement of these characters but if a character with low heritability (40% or less) selection may be comparatively difficult or virtually impractical due to masking effect of the environment on the genotypic effects. This indicated that selection for density of oil glands, albedo thickness, fruit weight and fruit diameter would be effective. In a similar study, Panse

(1957) suggested that a high genetic advance coupled with high heritability may be expected if heritability is mainly due to additive genetic effects.

Table 3. Variability, heritability and genetic advance in grapefruit and tangelo varieties based on morphological characteristics

Characters (Grapefruit)	PV	GV	PCV	GCV	h^2 (%)	GA (%) of mean
Leaf lamina length (mm)	79.5	63.4	8.4	7.5	79.7	13.9
Leaf lamina width (mm)	42.4	28.1	9.6	7.8	66.3	13.2
Rootstock bud circumference (cm)	148.3	145.4	12.5	12.3	97.9	25.2
Leaf area	16.1	14.3	14.8	13.9	88.5	27.1
Scion truck circumference	216.9	212.5	16.5	16.4	97.9	33.4
Average number of seeds per fruit	193.9	192.1	102.3	101.9	99.0	209.0
Seed weight per fruit (g)	190.7	189.4	104.1	103.7	99.3	213.0
Seed length (mm)	7.8	6.8	18.2	16.9	86.8	32.5
Seed width (mm)	1.5	1.4	17.9	17.2	92.3	34.1
Flower diameter (mm)	5.5	4.0	7.0	6.0	73.3	10.6
Staminate (%)	3.0	2.9	26.4	25.7	94.7	51.6
Perfect (%)	4.4	2.3	2.2	1.6	52.9	2.4
Pollen viability (%)	150.2	145.6	20.2	19.8	96.9	40.3
Pollen germination	47.9	45.8	17.5	17.1	95.5	34.5
Fruit diameter (mm)	246.6	235.7	16.2	15.9	95.5	32.0
Fruit length (mm)	110.8	102.3	12.0	11.5	92.3	22.9
Fruit rind thickness (mm)	3.6	3.2	24.3	22.7	87.1	43.7
Number of segments per fruit	13.0	8.9	23.7	19.5	68.1	33.2
Diameter of fruit axis (mm)	13.8	11.8	27.9	25.8	85.3	49.2
Total soluble solids (%)	0.4	0.3	8.4	6.9	67.1	11.7
Acidity (%)	0.1	0.1	40.4	38.8	92.1	76.7
Ph	0.1	0.0	8.8	5.4	37.1	6.7
VIT C	59.1	50.4	17.5	16.2	85.2	30.8
JUICE %	66.2	56.2	15.9	14.7	84.8	27.9
PEEL %	18.2	9.0	13.7	9.6	49.2	13.9
RAG %	42.4	19.0	36.4	24.4	44.8	33.6
Number fruits per tree	22200.2	22161.3	60.0	60.0	99.8	123.5
Fruit weight (g)	12060.5	11900.6	30.8	30.6	98.6	62.7

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

Allele amplification in grapefruit and tangelo varieties

Allele frequency or the frequency at which alleles are found at any locus of interest is used to estimate the frequency of given genetic profile. In grapefruit and tangelo varieties a total of 75 alleles were amplified by 26 polymorphic SSR loci and the number of alleles ranged from 1 to 4 with an average of 2.88 alleles per locus. The highest number of alleles per locus was four as amplified by CAT01, CS05, CCSM204,

CCSM70, CIBE5156, ATC09 and CMS26 followed by three alleles per locus each by CCSM77, CCSM156, CL11, OP29, CMS46, CMS09, CIBE4728, CMS3O and AG14 and the remaining markers amplified two alleles (*Table 4*).

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

S. no.	SSR marker	Monomorphic allele	Polymorphic allele	Total no. of alleles	Polymorphism (%)	PIC	Genetic diversity
1	CS05	1	3	4	75.00	0.69	0.76
2	CCSME15	0	2	2	100.00	0.30	0.33
3	OP571	1	1	2	50.00	0.49	0.55
4	CAT01	1	3	4	75.00	0.75	0.83
5	CCSM77	0	3	3	100.00	0.59	0.65
6	CCSM156	1	2	3	66.67	0.61	0.67
7	CCSM170	1	1	2	50.00	0.50	0.55
8	CCSM201	1	1	2	50.00	0.28	0.31
9	CCSM204	1	3	4	75.00	0.64	0.71
10	CCSM68	0	2	2	100.00	0.43	0.47
11	CCSM70	1	3	4	75.00	0.74	0.82
12	CCSME15	0	2	2	100.00	0.18	0.20
13	CIBE5156	1	3	4	75.00	0.74	0.83
14	CL11	1	2	3	66.67	0.51	0.56
15	CS06	1	1	2	50.00	0.17	0.18
16	CS09	1	1	2	50.00	0.41	0.45
17	CIBE5720	1	1	2	50.00	0.49	0.55
18	GT03	1	1	2	50.00	0.49	0.55
19	ATC09	1	3	4	75.00	0.68	0.75
20	0P29	1	2	3	66.67	0.38	0.42
21	CMS46	1	2	3	66.67	0.61	0.67
22	CMS09	2	1	3	33.33	0.60	0.67
23	CIBE4728	2	1	3	33.33	0.66	0.73
24	CMS26	1	3	4	75.00	0.68	0.75
25	CMS30	0	3	3	100.00	0.55	0.61
26	AG14	0	3	3	100.00	0.55	0.61
	Total	**22**	**53**	**75**	**1808.34**	**13.72**	**15.18**
	Mean	**0.85**	**2.04**	**2.88**	**69.55**	**0.53**	**0.58**

Polymorphic information content and percentage of polymorphism among grapefruit and tangelo varieties based on SSR markers

In Grapefruit and tangelo varieties the percentage of polymorphism of the 26 polymorphic markers ranged from 50 to 100 (*Table 4*). Among these, 6 exhibited 100 percent polymorphism; seven were having 75 %, four having 66.66 % and remaining had 50 %. Average polymorphism (%) of all the polymorphic primer pairs across all the varieties of grapefruit was 69.55. The PIC value which is a measure of allelic diversity

at a locus ranged from 0.17 (CS06) to 0.75 (CAT01) with an average value of 0.53. Fourteen SSR markers revealed PIC value more than 0.53. Primer CAT01 amplified 4 alleles and had the highest PIC value of 0.75 followed by CSM70 and CIBE5156 in which 3 alleles were amplified and had PIC value of 0.74 (*Table 4*). All the alleles amplified by CAT01 primer pairs on all the varieties of grapefruit were all distinguishable. It has been observed that marker OP29 amplified 3 alleles and had PIC value of 0.38 while GT03 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 663 alleles (*Table 5*) were amplified by 58 SSR primers with an average of 63.30 alleles for each variety. The average amplified fragments for polymorphic marker was 51.90 whereas for monomorphic, it was 14.40. The maximum number of alleles (75) was detected in Ray Ruby whereas Minneola showed the least number of alleles (52). However, the percent of polymorphic markers was maximum (82.09) in Marsh followed by 81.25 % in Pearl.

Table 5. *Total number of alleles amplified in grapefruit and tangelo varieties using SSR markers*

S. no.	Genotypes	Number of amplified alleles		Total	Polymorphism (%)
		Monomorphic markers	Polymorphic markers		
Grapefruit					
1	Foster	12	48	60	80.00
2	Flame	13	48	61	78.69
3	Marsh	12	55	67	82.09
4	Ray Ruby	15	60	75	80.00
5	Red Blush	20	52	72	72.22
6	Rio Red	16	56	72	77.78
7	Ruby Red	14	60	74	81.08
8	Star Ruby	15	51	66	77.27
Tangelo					
9	Pearl	12	52	64	81.25
10	Minneola	15	37	52	71.15
	Total	**144**	**519**	**663**	**781.53**
	Mean	**14.40**	**51.90**	**66.30**	**78.15**

Likewise, Meral et al. (2011) 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. Similarly, Singh et al. (2016) concluded that among 19 different mandarin genotypes that 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). 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.

Simple Sequence Repeats (SSRs) have proven to be efficient genetic markers for comparative genetic mapping between Citrus species (Luro et al., 2008). Although some SSRs were identified based on EST database in previous studies (Chen et al., 2006) no system analysis of SSRs in citrus has been reported because of incomplete citrus genome. Recently, the Clementine mandarin genome has been sequenced (Gmitter et al., 2012) and the completion of these genome sequences provided an opportunity for us to scan the entire genome for SSR discovery in citrus. In this study, we present our results on the SSR survey for the development of citrus SSR markers.

Genetic diversity

Genetic diversity among grapefruit and tangelo varieties ranged from 0.18 (CS06) to 0.83 (CAT01 and CIBE5156). The average value of genetic diversity across all the primers was 0.58 (*Table 4*). The dendrogram (*Fig. 2*) depicting the genetic relationship classified the genotypes into 3 major clusters (I, II and III). The cluster I contains single variety (Minneola) but cluster II was further sub divided into two sub clusters IIA and IIB with three (Rio Red, Star Ruby and Pearl) and two (Flame and Foster) varieties respectively. While four (Ray Ruby, Marsh, Ruby Red and Red Blush) varieties were clustered in cluster III. The similarity coefficient based on DNA amplification of grapefruit and tangelo varieties using SSR primer was estimated by dice similarity coefficient (*Table 6*). The varieties Ruby Flame and Foster, Ruby Red and Red Blush showed the highest genetic similarity having coefficient of 0.90 and were closely related. However, Rio Red and Pearl showed lowest (0.68) genetic similarity coefficient and these were genetically distinct from each other. Similarly, 19 different mandarin genotypes (Singh et al., 2016) 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 III. Clustering was according to the breeding history of genotypes but independent of their geographic origin.
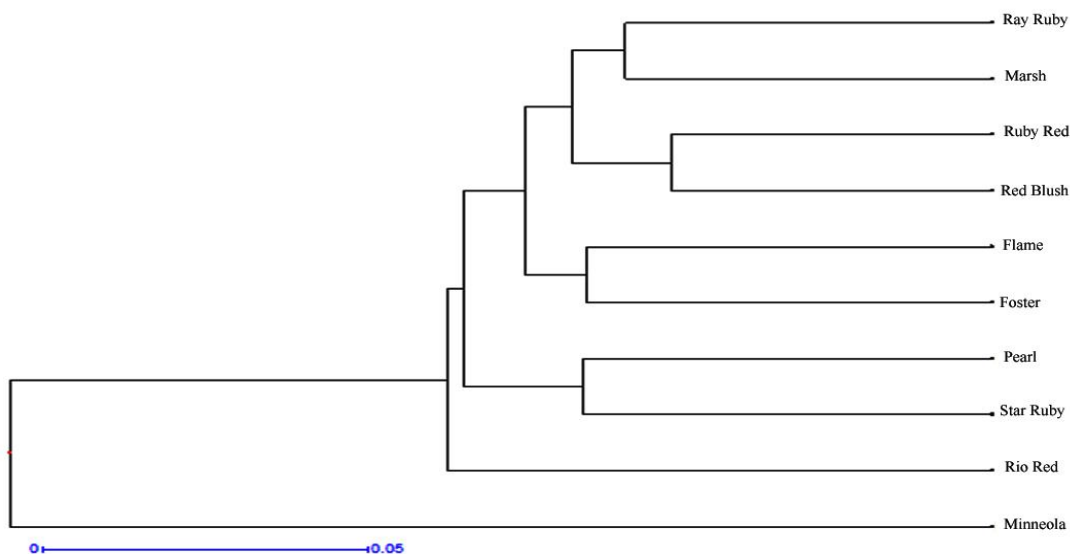

Figure 2. Dendrogram illustrating genetic relationship among grapefruit and tangelo varieties based on the SSR markers generated by UPGMA tree analysis

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Table 6. *Similarity coefficient based on DNA amplification of grapefruit and tangelo varieties estimated by dice similarity coefficient*

Varieties	1	2	3	4	5	6	7	8	9
2	0.76								
3	0.88	0.71							
4	0.85	0.73	0.87						
5	0.87	0.69	0.85	0.89					
6	0.83	0.76	0.83	0.83	0.86				
7	0.82	0.68	0.82	0.82	0.85	0.83			
8	0.87	0.73	0.87	0.89	0.90	0.90	0.87		
9	0.85	0.71	0.81	0.81	0.83	0.84	0.83	0.88	
10	0.86	0.68	0.82	0.82	0.84	0.83	0.84	0.86	0.87

Legend: Marsh, Ray Ruby, Rio Red, Star Ruby, Flame, Red Blush, Ruby Red, Foster, Minneola, and Pearl

The results revealed that the difference in the dendrogram of morphological and molecular data could be due to action of diverse evolutionary forces and environmental attributes. This hypothesis was supported by the findings of Paudyal and Haq (2008) who reported that environmental factors affected up to 40% in pummelo accessions in an un-controlled field survey. The morphological difference in individual accession was supported by the observation of Dorji and Yapwattanaphun (2011a) who reported that phenotypic variation could be attributed to mutations, cross pollination and environmental interactions (*Figs. 3, 4* and *5; Table 7*).

Table 7. *Physico-chemical parameters of grapefruit and tangelo varieties*

Varieties	Average number of seeds per fruit	Seed weight per fruit (g)	Rootstock diameter (mm)	Scion diameter (mm)	Spine length (mm)	Leaf lamina length (mm)	Leaf lamina width (mm)	Leaf area (cm^2)	Petiole wing width (mm)	Flower diameter (mm)	Pollen viability (%)	Pollen germination (%)
Grapefruit												
Flame	3.3	2.3	94.3	90.1	5.6	117.6	76.9	32.7	12.9	32.8	51.4	32.9
Foster	34.5	30.2	83.2	80.3	5.7	105.9	66.1	27.7	16.3	35.0	54.9	38.2
Marsh Seedless	3.2	2.9	104.6	100.6	4.8	110.1	68.5	29.8	18.8	31.3	50.2	29.9
Ray Ruby	4.5	3.6	110.5	105.1	4.3	114.2	74.1	31.6	22.0	37.1	55.9	39.7
Red Blush	3.8	3.1	115.1	112.2	6.3	104.2	64.7	25.3	15.6	33.1	57.6	40.4
Rio Red	1.8	1.1	108.0	96.1	5.1	99.1	65.2	23.9	17.8	31.6	53.2	36.6
Ruby Red	3.5	3.3	87.3	74.1	5.5	97.0	73.3	23.1	14.5	35.6	53.5	34.7
Star Ruby	35.8	33.1	79.5	65.6	4.3	110.9	63.3	30.4	19.8	34.0	66.7	42.4
Tangelo												
Minneola	21.5	24.7	92.0	80.1	3.4	101.7	61.1	24.5	23.3	30.4	77.7	46.9
Pearl	24.0	28.3	98.7	84.5	3.6	90.7	60.0	21.7	20.7	32.2	85.7	53.0
SEm±	2.3	1.9	1.0	1.2	0.6	6.9	6.5	2.4	0.9	2.1	3.7	2.5
LSD (p=0.05)	0.8	0.7	3.0	3.6	0.2	2.3	2.2	0.8	0.3	0.7	1.2	0.8

| Varieties	Fruit diameter (mm)	Fruit length (mm)	Fruit rind thickness (mm)	Number of segments per fruit	Total soluble solids (Brix)	Acidity (%)	pH	Ascorbic acid (mg/100g)	Juice content (%)	Peel content (%)	Rag content (%)	Fruit weight (g)
Grapefruit												
Flame	93.0	83.7	7.2	12.8	7.3	1.13	4.1	52.9	52.3	34.9	12.8	258.9
Foster	99.1	91.3	8.5	15.4	8.7	0.90	4.2	40.9	45.6	33.4	21.2	431.9
Marsh Seedless	103.1	93.9	8.5	12.6	7.5	1.45	4.5	54.7	46.8	32.8	20.5	337.1
Ray Ruby	111.6	96.9	7.7	14.4	7.9	1.80	5.0	50.9	48.4	31.3	20.2	384.0
Red Blush	84.9	75.9	10.4	13.7	8.2	0.62	4.8	35.8	51.9	31.5	16.6	313.7
Rio Red	114.4	98.8	9.7	15.4	8.2	1.16	4.4	41.5	44.0	32.1	23.9	456.5
Ruby Red	109.1	97.5	9.3	13.0	8.5	1.03	4.8	43.9	40.4	34.4	25.2	561.8
Star Ruby	104.9	87.0	7.5	13.5	8.0	0.78	4.3	46.6	53.7	30.7	15.6	359.4
Tangelo												
Minneola	70.5	68.0	4.4	19.5	9.4	0.54	4.7	36.9	64.3	26.7	9.0	203.6
Pearl	74.0	81.1	5.6	22.3	8.4	0.64	4.8	34.0	62.5	23.7	13.8	236.2
SEm±	5.7	5.0	1.2	3.5	0.7	0.19	0.6	5.1	5.5	5.3	8.4	21.9
LSD (p=0.05)	1.9	1.7	0.4	1.2	0.2	0.07	0.2	1.7	1.8	1.8	2.8	7.3
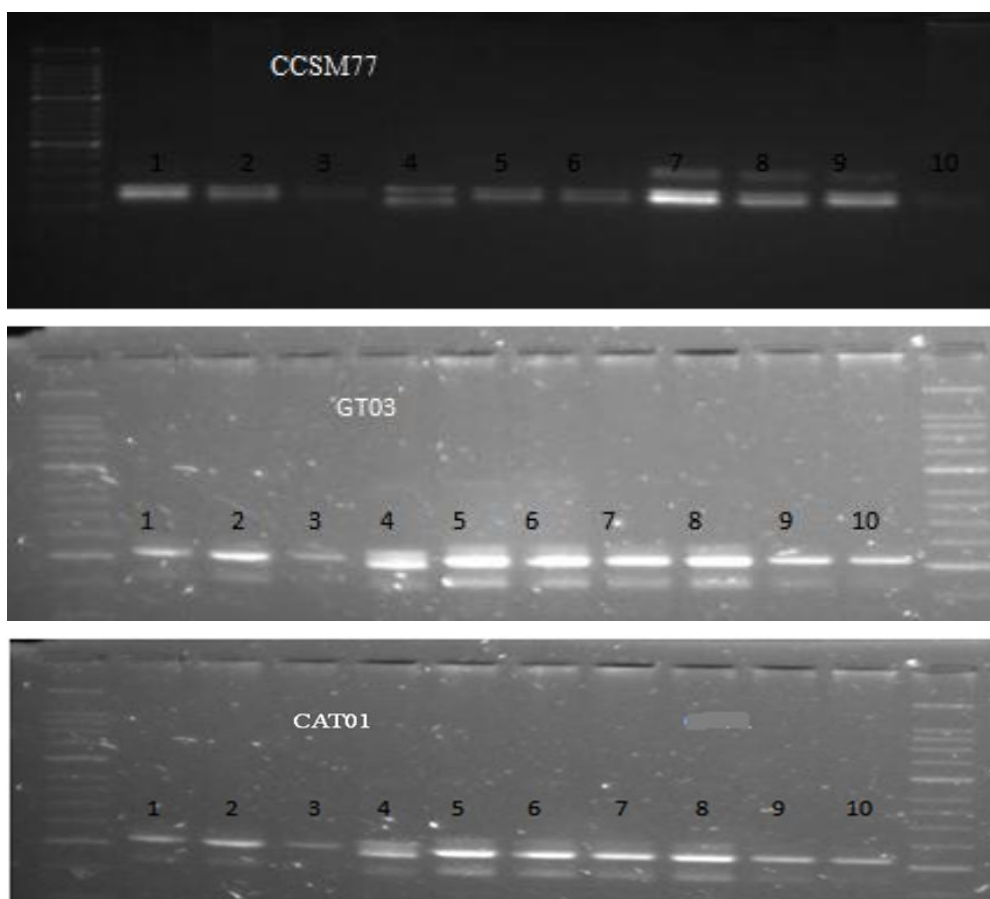
Figure 3. *Agarose gel showing SSR amplification profile by different primer in different grapefruit and tangelo varieties Flame, Foster, Marsh, Ray Ruby, Red Blush, Rio Red, Ruby Red, Star Ruby, Minneola and Pear*
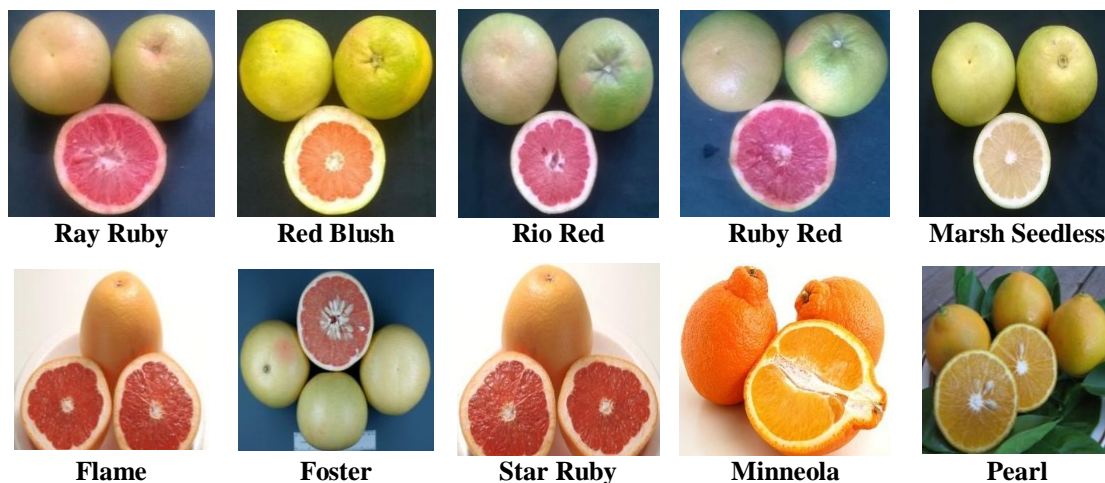
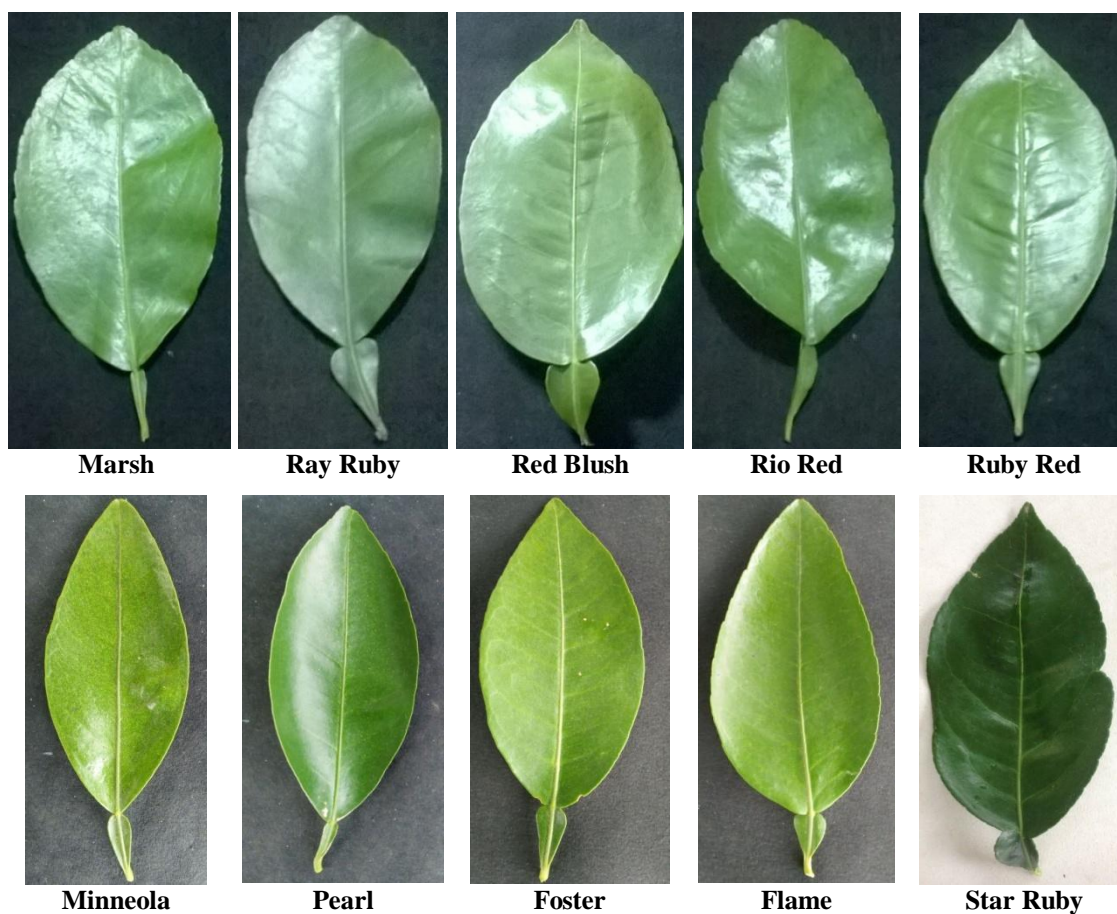**Figure 4.** *Diversity in fruit size and shape of different grapefruit and tangelo varieties*

Figure 5. *Diversity in leaf size and shape of different grapefruit and tangelo varieties*

Conclusions

Present study indicated that genetic diversity in grapefruit and tangelo varieties was found to be very low, 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. Genetic diversity among grapefruit and tangelo varieties ranged from 0.18 (CS06) to 0.83 (CAT01 and CIBE5156). Furthermore, based on molecular analysis, varieties Ruby Flame and Foster, Ruby Red and Red Blush showed the highest genetic similarity having coefficient of 0.90 and were closely related. However, Rio Red and Pearl showed the lowest (0.68) genetic similarity coefficient and these were genetically distinct from each other.

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