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Characterization of citrus germplasm using simple sequence repeat (SSR) markers

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Abstract

In the present study, 25 simple sequence repeat markers were used to detect molecular polymorphism among 30 citrus genotypes representing seedling selections, clonal selections, exotic and indigenous species. A total of 87 alleles were detected and the banding pattern resolved by each primer pair was in accordance with single locus variation. Out of 25 primer pairs, 20 were found polymorphic and produced alleles ranging between 2 to 6 with an average value of 3.95. The polymorphism information content ranged from 0.16 for DY263095 to 0.39 for DY289396. Out of 20 SSR markers, 10 SSRs amplified specific alleles among eight genotypes. The genetic similarity coefficient for all accessions ranged from 0.11-0.85%. Cluster analysis separated the genotypes into two groups at 0.11% similarities. The first major group comprised four species (G-26, G-27, G-29 and G-30). The 2nd major group consisted of 26 genotypes. Results confirmed that seedling origin pummelo (G-1 to G-5) are not true pummelo possibly because of their hybrid origin. However, seedless clone (G-18) and early bearing clone (G-20) of Nagpur mandarin showed similarity (78% and 69%, respectively) with Nagpur mandarin. The dwarf cluster bearing probable citrus hybrid exhibited affinity with sweet lime and constituted a group with 54% similarity.

Key words: Citrus, germplasm, molecular charachterization, simple sequence repeat markers

Introduction

Citrus and its close relatives are represented by 28 genera in the tribe Citreae of the subfamily Aurantioideae in the family Rutaceae [1]. Before the advent of molecular markers, citrus was classified based on morphology or biochemical techniques such as isozymes. Phylogeny and taxonomy for certain citrus cultivars have been somewhat debatable in the past; however, results from molecular marker technologies are helping to clarify some of these relationships [2]. The difficulty in classifying citrus is mainly due to sexual compatibilities between citrus and related genera, the high frequency of bud mutations, the long history of cultivation, wide dispersion and adventitious nucellar embryony which stabilizes and perpetuates hybrid taxa [3]. Understanding genetic variability in citrus is critical for determining genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies of core collections, establishing breeding programs, and the registration of new cultivars [4].

Several previous studies have utilized various molecular markers *viz*., ISSR, RAPD, AFLP and SSR to fingerprint, evaluate phylogenetic relationships and examine the level of genetic diversity among accessions in citrus. Many of these studies have targeted species, citrus groups or sampled a few individuals of each taxon. Bretó *et al*. [5] examined the variability of 24 Clementine (*C. reticulata* Blanco) accessions by utilizing ISSR, RAPD, and AFLP markers and found that only two varieties of 24 could be distinguished. Gulsen and Roose [6] used ISSR, SSR and isozymes to measure genetic diversity and phylogenetic relationships among 95 citrus accessions including 57 lemons (*C. limon* Burm.), related taxa, and three proposed ancestral species, *C. maxima* Burm., *C. medica* L. and *C. reticulata* Blanco. Five isozyme and five SSRs loci revealed relatively little variation among most lemons, but a high level of variation among the relatively distant citrus taxa. All the categories of lemons including rough and sweet lemons as well as some other suspected hybrids have

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been clustered with citrons. Most lemons (68%) had nearly identical molecular genotype, suggesting they originated from a single clonal parent via a series of mutations. The study employing isozymes, RFLP and ISSR markers classified 48 trifoliate orange (*Poncirus trifoliate* L. Raf.) accessions into four groups [7]. Fang and Roose [8] utilized ISSR markers to distinguish closely related cultivars, many of which had arisen through selection of spontaneous mutations. This study showed that ISSR markers could distinguish some (but not all) of these closely related accessions. Nicolosi *et al*. [9] used RAPD, SCAR, and cpDNA markers to elucidate phylogenetic relationships and genetic origins of hybrids in 36 accessions of citrus and one accession from each of four related genera. Federici *et al*. [10] examined the phylogenetic relations of 88 accessions representing 45 citrus species and six related genera by utilizing RFLP and RAPD markers. Overall, these previous studies demonstrated that molecular markers are powerful tools for elucidating genetic diversity, determining parentage, and revealing phylogenetic relationships among various citrus species; however, accessions arising from spontaneous mutation are often difficult to distinguish.

Simple sequence repeats (SSRs), a type of microsatellite marker, are particularly useful for characterization of germplasm collections because they are highly polymorphic and usually codominant [11-15], but they have not been widely used in citrus. The objective of this study was to use 25 SSR markers to detect polymorphisms among 30 citrus genotypes and their near relatives to determine the level of genetic diversity within this collection.

Materials and methods

Citrus genotypes

A total of 30 citrus genotypes representing seedling collections, clonal selections, exotic and indigenous species were used in the present study. These genotypes were obtained from the Division of Fruits and Horticultural Technology, Indian Agricultural Research Institute, New Delhi. The genotypes chosen from the citrus and its related genus consisted of 10 pummelo variants, two Nagpur mandarin variants, flying dragon (*Poncirus trifoliata*), Troyer (*Citrus sinensis* x *Poncirus trifoliata*), Alemow (*Citrus macrophylla*), Rough lemon (*Citrus jambhiri*), Rangpur lime (*Citrus limonia*), Galgal (*Citrus pseudolimon*), sweet lime (*Citrus limmetoids*), dwarf cluster hybrid of unknown origin, hybrid between Galgal and Pummelo, *Citrus megaloxycarpa*, *Citrus macroptera*, *Citrus latipes*, *Citrus assamensis* (Assam lemon), *Citrus jambhiri* (Kachai lemon), *Citrus indica* (Indian wild orange), *Citrus penivesiculata (*Adajamir) and *Citrus rugulosa* (Atanni) (Table 1).

DNA isolation, PCR and gel electrophoresis

A total of 25 primer pairs, synthesized by M/s. Sigma (USA) were used for PCR amplification. These primers were selected based on their uniform distribution across the genome and details of the primers used are given in the Table 2. The genomic DNA of different citrus genotypes was isolated from young leaves and purified following the standard protocol [16] and the quality and quantity of DNA was estimated using a UV spectrophotometer (Beckman, USA). Genomic DNA samples were diluted to 30 ng/µl and were subjected to polymerase chain reaction. The PCR was conducted in a total reaction volume of 20 µl per sample, containing 10× reaction buffer 2µl (50mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 9.0), dNTPs (1.25µM each) 2µl, forward microsatellite primer 0.15µl (200µM), reverse microsatellite primer 0.15µl (200µM), *Taq* polymerase (3U/µl) 0.15µl and DNA 50-100mg. This reaction mixture was processed in a programmable Thermal Cycler, Gene Amp PCR System 9700 (Perkin Elmer Applied Biosystem, USA), programmed for 35 cycles for 1 min at 94°C, 1 min at 55-58°C, 2 min at 72°C with initial denaturation for 5 min at 94°C and final extension for 7 min at 72°C. After amplification, the amplified products were mixed with $1/6^{th}$ volume of the gel loading cum tracking dye (40% sucrose: 0.25% bromophenol) and loaded onto each well of 3.5% metaphore gel and run at a constant voltage of 120 volts for 3 hrs. The bands were visualized using Alpha Image 1220 and documented.

Data analysis

The amplified DNA fragments were scored as present (1) or absent (0) for each primer genotype combination. The data was entered into a binary matrix and subsequently analysed using the computer package NTSYS-pc Version 2.02 [17]. Dice similarity coefficients were calculated and used to ascertain the genetic interrelationship by (1) partitioning the variance of the data sets using principal component analysis (PCA); (2) Constructing phonetic tree using UPGMA (Unweighted Pair Group Method of Arithmetic mean) cluster analysis. PIC expresses the discrimination power of the locus by taking into account not only the number of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus.

Table 1. List of genotypes used in the study

S.No.	Genotype code	Remarks
1.	G-1	Probable pummelo (*Citrus grandis* Osbeck) hybrid
2.	G-2	Probable pummelo, seedling selection from north east India.
3.	G-3	Red fleshed seedling selection of pummelo.
4.	G-4	Intermediate pummelo type, red and white coloured segments, tender in eating.
5.	G-5	Pummelo selection, red fleshed, sweet, medium size fruits.
6.	G-6	Clonal selection pummelo from Nagpur
7.	G-7	-do-
8.	G-8	-do-
9.	G-9	-do-
10.	G-10	-do-
11.	G-11	Flying Dragon (*Poncirus trifoliata* (L.) Raf.).
12.	G-12	Rough Lemon (*Citrus jambhiri* Lush.).
13.	G-13	Troyer (hybrid of *Citrus sinensis* (L.) Osbeck *x Poncirus trifoliata* (L.) Raf.).
14	G-14	Alemow (*Citrus macrophylla* Wester).
15.	G-15	Rangpur lime (*Citrus limonia* Osbeck).
16.	G-16	Galgal (*Citrus* pseudolimon Tanaka) selection.
17.	G-17	Sweet lime (*Citrus limettioides* Tanaka) selection from Uttarakhand.
18.	G-18	Mandarin-4, seedless selection from Nagpur mandarin (*Citrus reticulata* Blanco).
19.	G-19	Nagpur mandarin (*Citrus reticulata* Blanco).
20.	G-20	Mandarin-2, early bearing clone of Nagpur mandarin.
21.	G-21	Hybrid of Galgal (*Citrus pseudolimon* Osbeck x Pummelo (*Citrus grandis* Osbeck).
22.	G-22	Dwarf cluster bearing hybrid of unknown origin.
23.	G-23	Sour pummelo (*C. megaloxycarpa* Lush.).
24.	G-24	Metanewsian Papeda (*Citrus macroptera* Tanaka).
25.	G-25	Khasi Papeda (*Citrus latipes* Tanaka).
26.	G-26	Assam lemon (*Citrus assamensis* Dutta and Bhattach.).
27.	G-27	Kachai lemon (*Citrus jambhiri* Lush.).
28	G-28	Indian wild orange (*Citrus indica* Tanaka).
29.	G-29	Adajamir (*Citrus penivesiculata* var. assamensis Lush.).
30.	G-30	Atanni (*Citrus rugulosa* Tanaka).

Fig. 1. PCR profile of SSR marker DY 265504 in the citrus genotypes. Lane M: DNA marker standard (100 bp), lanes 1 to 30: citrus genotypes in the same order as presented in Table 1

Table 2. List of polymorphic SSR primers used for the assessment of genetic diversity in *Citrus*

S.No	Primer	Annealing temp.	Alleles amplified	PIC values
1	DY264355	55	4	0.18
2	DY294759	55	3	0.22
3	DY263095	58	2	0.16
4	DY264633	58	2	0.17
5	DY280390	58	6	0.20
6	DY287851	55	5	0.23
7	DY294129	58	5	0.20
8	DY284947	58	3	0.31
9	DY265504	55	5	0.17
10	DY277386	55	3	0.24
11	DY274485	58	4	0.26
12	DY296883	55	5	0.24
13	DY292105	58	5	0.29
14	DY275927	58	4	0.22
15	DY284275	55	4	0.19
16	DY289396	58	3	0.39
17	CAC39	55	5	0.28
18	Cac23	55	5	0.31
19	AG14	55	3	0.29
20	TAA1	55	3	0.22

Polymorphism information content (PIC) was calculated as per the standard procedure [18].

Results and discussion

Microsatellite polymorphism

A total of 87 alleles were identified with the 25 primer pairs in the 30 citrus genotypes, and the banding pattern resolved by each primer pair are in accordance with single locus variation. Out of 25 primer pairs, 20 were found polymorphic and produced alleles ranging between 2 to 6 with an average value of 3.95, which was lower than the average value obtained by Ahmed *et al*. [19] and Barkley *et al*. [20] The possible reason for this difference may be the higher both SSR markers applied and accessions analyzed. The polymorphism information content for these 20 primer pairs ranged from 0.16 for DY263095 to 0.39 for DY289396. The results of 20 microsatellites along PIC values are given in Table 2.

Genotype specific allele

Out of 30 genotypes used in the study, eight genotypes namely, Pummelo 9 (G-9), Alemow (G-14), Galagal x Pummelo hybrid (G-21), *Citrus macroptera* (G-24), *Citrus latipes* (G-25), Assam lemon (G-26), Adajamir (G-29) and Atanni (G-30) genotypes were amplified specific alleles using 10 different SSR markers (Table 3). Moreover, in galgal x pummelo probable hybrid two SSRs *viz*., CAC23 and TAA1 resulted in amplification of specific alleles. Similarly, in Assam lemon (DY292105 and DY265504) and Atanni (DY284275 and DY287851) two different SSRs resulted in specific alleles amplifications (Table 3).

PCA and dendrograme analysis

Principal component analysis of the citrus microsatellite data from 20 primer pairs separated the G-1 to G-5 from rests all of the citrus genotypes by the first and second principal component. The first two components having eigen values >1 explained about 37% of the variation and thus the total of variability is not explained by the first three components. Based on the projection on lines, genotypes belonging to another cluster G-6 to G-10 were fall in one component and make a separate group.

A dendrograme derived from UPGMA cluster analysis based on the dice similarity coefficient matrix for 30 genotypes was constructed. The genetic similarity coefficient for all accessions ranged from 0.11-0.85%. Cluster analysis separated the genotypes into two groups at 0.11% similarities (Fig. 2). The first major group comprised four genotypes (Assam lemon (G-26), Kachai lemon (G-27), Adajamir (G-29) and Atanni (G-30). Among these, three genotypes (Kachai lemon, Adajamir and Atanni formed sub group at 0.27% similarity and one genotypes i.e. Assam lemon was separated as a subgroup 2. The 2^{nd} major group consisted of 26 genotypes. From the major group, seedling origin pummelo collections such as G-2 and G3 collected from hot spot of citrus growing in India (North East), G-1 assumed to be hybrid of pummelo, G-4 intermediate type of pummelo having red and white coloured tender segments and G-5 pummelo selection having red flesh, medium sized sweet fruits formed a separate group (G-1 to G-5). Interestingly, this group showed only 20% similarity with the clonal selections of pummelo taken in this study (G-6 to G-10). However, seedling origin pummelo (G-1 to G-5) had >40% similarity with Rangpur Lime (G15). Moreover, G-2, G-4 and G-5 had more than 50% similarity with Rangpur lime (G-5). Our results thus confirmed that seedling origin pummelo (G-1 to G-5) are not true pummelo

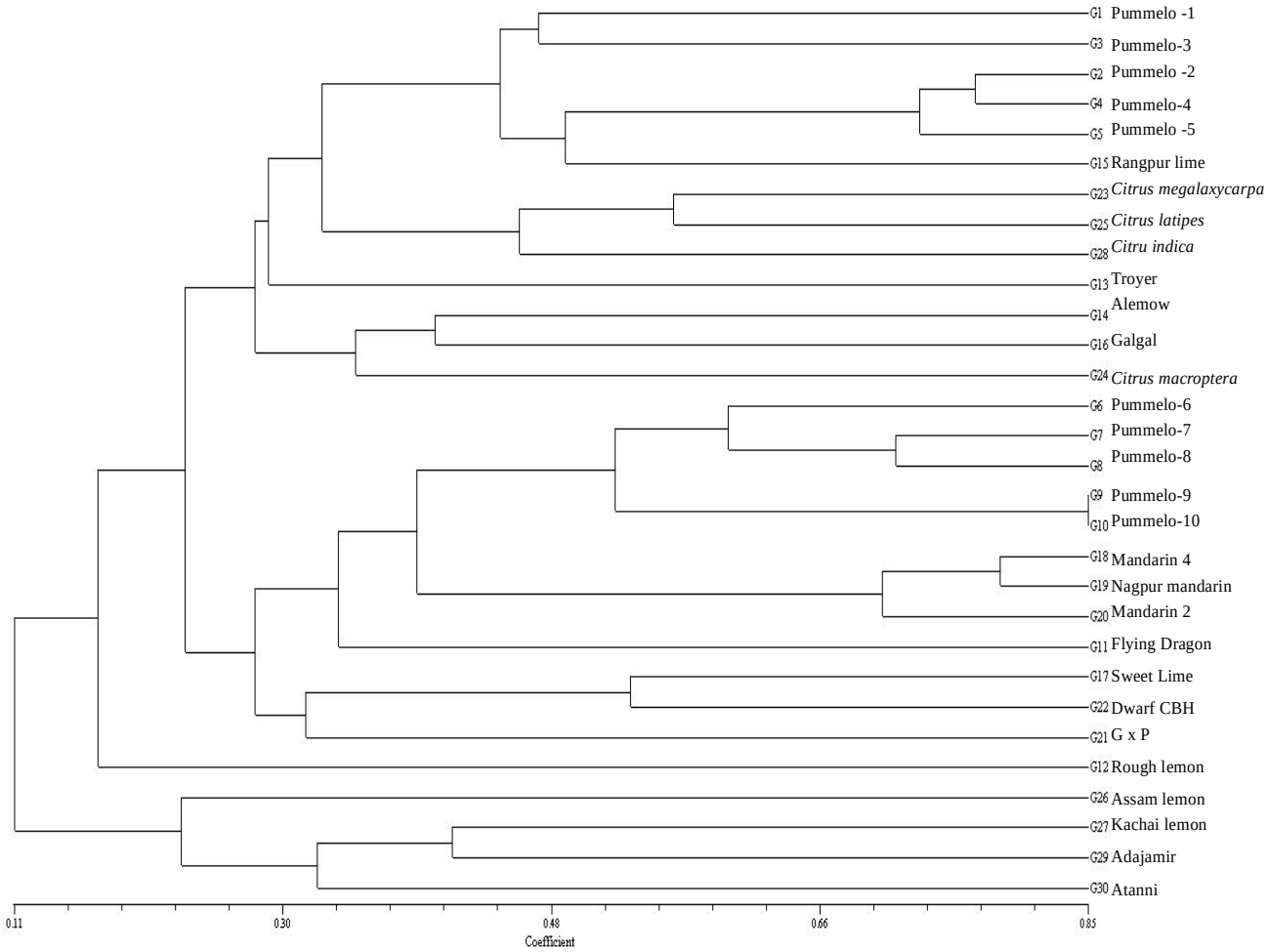

Fig. 2. Dendogramm of citrus species based on UPGMA cluster analysis

possibly because of their hybrid origin, moreover genome affinity of Rangpur lime (G-15) in the origin of these probable pummelo seedling type collections can not be ruled out.

It was also evident that *Citrus megaloxycarpa* (G-23), *Citrus latipes* (G-25) and *Citrus indica* (G-28) formed a separate group and showed 30% similarity with probable pummelo seedling type collections. These citrus species were included in the study with the hypothesis that *Citrus megaloxycarpa* is a hybrids of pummelo [21]. However, Singh and Nath [22] contradicted the findings of Lushington [21] and designated this as *Citrus grandis.* The results obtained in the present study by using SSR markers clearly revealed that seedling type pummelo collections are not true pummelo and showed very less affinity with *Citrus megaloxycarpa*. Furthermore, the clonal pummelo selection (G-6 to G-10) formed a separate group at 50% similarity and G-9 and G-10 showed similarity more than 85% and grouped in the same sub-cluster.

The variants of Nagpur mandarin like G-20 an early bearing clone of Nagpur mandarin, ripens early under Nagpur conditions and G-18 a seedless selection of Nagpur mandarin formed a separate sub-cluster with Nagpur mandarin (G-19). The similarity between Nagpur mandarin and seedless Nagpur mandarin selection (G-18) was about 78%. However, early bearing Nagpur mandarin selection (G-20) showed only 69% similarity with Nagpur mandarin and seedless Nagpur mandarin selection. The dissimilarity between variants of Nagpur mandarin with original Nagpur mandarin may be attributed to the changes at genetic level at some point of time resulting seedlessness and early bearing attributes. Our results are in agreement to the earlier findings [23] which showed that RAPD profile reveals variation among elite clones of mandarin. The probable

Table 3. List of SSR primers amplified genotypes specific alleles

S.No.	Genotypes	Primers
1	G-9	DY294129
2	G-14	DY280390
3	G-21	Cac23 & TAA1
4	G-24	DY292105
5	G-25	CAC39
6	G-26	DY292105 & DY265504
7	G-29	DY265504
8	G-30	DY284275 & DY287851

dwarf statured hybrid citrus of unknown origin (G-22) showing tendency of cluster bearing had 50% affinity with sweet lime (G-17) and formed an independent group. The similarity between dwarf cluster bearing hybrid with sweet lime confirms hybrid origin of G-22 and role of sweet lime in its origin is ascertained.

Another collection G-21, which was suspected to be a hybrid of Galgal x Pummelo showed 30% similarity with sweet lime and dwarf cluster bearing citrus genotype and 28% similarity with clonal pummelo collections. Interestingly, this genotype showed high degree of dissimilarity (>80%) with original galgal in present molecular analysis. It is evident from the present SSR study that in the evolution of G-21, pummelo might have minor but important roles than galgal. A few indigenous species of citrus like Assam lemon, kachai lemon, Adjamir and Atanni were also taken in the study to discover their contribution in the origin of seedling type pummelo and to assess the molecular affinity of theses species with other citrus species. It was found that Assam lemon, Kachai lemon, Adjamir and Atanni formed a separate group and had only 10% similarity with the rest of citrus genotypes used in the present investigation.

It can be concluded from the present investigation that SSRs are strong molecular markers to characterize citrus germplasm. The genetic characterization of citrus genotypes using SSR makers ascertained that citrus genotypes assumed to be pummelo are not true pummelo possibly because of their hybrid origin. Seedless and early bearing clones of Nagpur mandarin had close similarity with Nagpur mandarin and sweet lime which is likely to have contributed in the origin of dwarf cluster bearing hybrid of unknown origin.

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