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DNA fingerprinting in citrus for species identification

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Abstract
In the present investigation, A total of twenty-one SSR primers were used to evaluate the genetic diversity of six citrus species. Seven primer pairs failed to show any amplification thus revealing no bands (null allele) in all the genotypes. Fourteen SSR markers exhibited polymorphism and showed high levels of allelic diversity. A total of 139 amplicons were amplified by 14 polymorphic SSR loci and the number of amplicons ranged from 5 to 18 with an average of 9.92 amplicons per locus. Only one primer i.e. SSR-5 shown 100 % polymorphism, whereas, 87 % polymorphism was observed in SSR-2 and 67 % polymorphism was detected in SSR-3, SSR-9, SSR-12 and SSR-13; showed an average 66.14 % polymorphism percent. The PIC value of 14 microsatellite loci ranged from 0.41 to 0.86 with an average value of 0.59. For each marker, the maximum PIC value was observed in marker SSR-5 i.e. 0.86 and the minimum was in SSR-11 i.e. 0.41. The highest similarity was found between Alemow and Galgal with a correlation coefficient value of 0.64. The lowest similarity coefficient was observed between Jambhiri and Galgal with a similarity coefficient value of 0.44.

Keywords: Galgal, rootstock identification, SSR, Molecular characterization

Introduction
Citrus is the most important group of fruit commercially cultivated on a worldwide basis and is grown wherever the climate is suitable. It is widely grown in most areas with suitable climates tropical, subtropical and borderline subtropical and temperate condition (Kahn *et al*. 2001) [6].
Citrus is one of the most remunerative fruit crops of India, having a lasting niche in international trade and world finance. Hence, it occupies an important place in the wealth and economy of India, as the third-largest fruit industry after Mango and Banana.
Citrus is growing in more than 142 countries; major commercial citrus growing countries by areas and production include southern China, followed by Nigeria, Colombia and then India. India is contributing 4.48 percent of production in the world. In India, Maharashtra, Andhra Pradesh, Karnataka, Punjab, Tamil Nadu, Assam, Meghalaya, West Bengal, Sikkim, and Haryana are major citrus-producing states. These states contribute estimated production of 13,976 thousand tonnes and an area covered of about 1054 thousand acres in 2019-20 (Source, NHB).
Commercial citrus cultivation has been done by grafting and budding. The rootstock is a very important part of a citrus orchard. It has varied effects on scion vigour and size, fruit yield, tolerance to various biotic and abiotic stresses. It is therefore of utmost importance to select the best performing rootstock for a given variety in a given region to attain maximum productivity.
Rough lemon (*C. jambheri* Lush.) and Rangpur lime (*C. limonia*) are the widely used rootstocks in India, due to their resistance to gummosis and root rot (Sonkar, 2001) [15]. Sometimes inadvertent seed mixtures of related species are the big problem for citrus grower. In India, the largest area under citrus cultivation is in the Vidarbha region of Maharashtra where farmers totally rely on the public and private nurseries for supply of planting material. Many nurseries unfortunately do not maintain the mother plants of the Rangpur lime (*Citrus limonia*) and Rough lemon (*Citrus jambhiri*) rootstocks and import seeds from the Himalayan foothill states. These rootstock seeds are randomly collected from different citrus species, particularly Galgal (*Citrus pseudolimon*). Although scions grafted on Galgal are vigorous and healthy, they are susceptible to *Phytophthora* and have a lesser life span thus requiring replanting of the orchard after six or seven years, thus it leads to the huge economic losses for a farmer.
A variety of methods have been used for citrus cultivar identification. (Luro *et al*. 1995) [7].

The conventional method of citrus cultivar identification relied on morphological features and isozymes. Using morphological traits, it is difficult to distinguish between many citrus cultivars because some cultivars are distinguishable only by fruit traits and citrus trees usually do not bear fruits until 3-4 years after planting. Moreover, isozyme markers can be mediated by secondary processes so that the normal patterns of expression are suppressed. (Atiyah, 2016) [1]. DNA fingerprinting is the technique used for identification of individual on the basis of their respective DNA profiles. It offers a faster and more precise way of determining relationships among closely related species than that of morphological investigation because morphological characteristics are subject to environmental influence (Rahman, 2007) [10]. Molecular techniques such as RAPD, RFLP, AFLP, SCAR and Microsatellite markers have been used to identify citrus species with high accuracy. SCARs marker is highly reliable, co-dominant and usually single locus and species specific. (Bhagyawant, 2015) [15].

In the present paper, we report the use of species specific SSR markers to identify Galgal rootstock from five different species under study.

Materials and Methods

Plant material

A total six species of citrus used in this study were collected from All India Co-ordinated Research Project on Citrus, P.D,K.V. Akola and Central Citrus Research Institute, Nagpur.

DNA isolation

Genomic DNA of six citrus species were extracted from young leaves using CTAB method as described by Cheng *et al*. (2003) [4] with minor modifications. The extraction buffer contained 100mM Tris, 25 mM EDTA, 1.4 M Nacl, 2% CTAB, 1% PVP and 0.2% β - Mercaptoethanol. The DNA obtained by extraction was confirmed by running on 0.8% agarose gel electrophoresis system. The extracted DNA was stored at -20^{0}C until use.

PCR amplification

Twenty one species specific SSR primer were designed and used for identification of different citrus species. The PCR amplification was performed in a 20 µl reaction volume containing 50 ng of template DNA, 1µl of single primer, 2.5 µl of 10x Taq buffer ($Mgcl_2$), 1µl of dNTP mixture and 0.3µl of Taq polymerase enzyme and the remaining was filled with deionized distilled water. Amplifications were carried out using a Thermocycler with an initial denaturation step of 5min at 94 °C, followed by 35 cycles of 1min at 94 °C, 45 sec at annealing temperature 65-68 °C and 1 min extension at 72 °C. A final extension step for 10 min at 72 °C was included. PCR products were separated by electrophoresis in 10% polyacrylamide gel with silver staining for detection.

Data analysis

The amplified products were scored for the presence (1) or absence (0) of bands of various sizes across the six different citrus species to generate a binary matrix. The weak and smeared fragments were not scored. The UPGMA Dendrogram was constructed using Jaccard's similarity coefficient.

Results and Discussion

In the present study, a total of twenty-one SSR primers were used to evaluate the genetic diversity of six citrus species. Seven primer pairs failed to show any amplification thus revealing no bands (null allele) in all the genotypes. Fourteen SSR markers exhibited polymorphism (Table 1) and showed high levels of allelic diversity. A total of 139 amplicons were amplified by 14 polymorphic SSR loci and the number of amplicons ranged from 5 to 18 with an average of 9.92 amplicons per locus.

Somewhat similar results were put forth by Ghorabaie *et al*. (2010) [5] who stated that Molecular markers would help to infer their relations with known cultivars. They used 30 Citrus accessions and eight known cultivars from the Kotra collection and investigated using 11 pairs of SSR markers, which in total produced 63 alleles with an average of 5.72 alleles per locus. The lowest number of alleles were observed in the cAGG9 locus with only 2 alleles and the highest number of alleles were observed in the TAA41 locus with 10 alleles.

Table 1: SSR primers used in the study and their PIC values

Sr. No	SSR primers	No. of amplicons	Monomorphic bands	Polymorphic bands	Polymorphism (%)	PIC value
1.	SSR-1	13	02	11	85	0.70
2.	SSR-2	16	02	14	87	0.68
3.	SSR-3	18	04	12	67	0.71
4.	SSR-4	10	02	08	80	0.70
5.	SSR-5	06	00	06	100	0.86
6.	SSR-6	05	03	02	40	0.42
7.	SSR-7	07	05	02	30	0.44
8.	SSR-8	12	02	10	83	0.71
9.	SSR-9	06	02	04	67	0.61
10.	SSR-10	10	04	06	60	0.43
11.	SSR-11	07	04	03	43	0.41
12.	SSR-12	12	04	08	67	0.64
13.	SSR-13	09	03	06	67	0.61
14.	SSR-14	08	04	04	50	0.44
	Total	139	41	96	926	
	Average	9.92	2.92	6.85	66.14	0.59

Polymorphic Information Content and percent polymorphism

Twenty-one SSR primers were used to detect the genetic diversity of citrus species. Seven primer pairs failed to show any amplification thus revealing no bands in all the genotypes. One primer SSR-5 shown 100 % polymorphism, whereas, the 87 % polymorphism observed in SSR-2, 67 % polymorphism was detected in SSR-3, SSR-9, SSR-12 and SSR-13; showed an average 66.14 % polymorphism percent. Total alleles per locus were 9.92, whereas, the average number of monomorphic and polymorphic alleles are 2.92 and 6.85, respectively. The PIC (Polymorphic information content) of 14 microsatellite loci ranged from 0.41 to 0.86 with an average value of 0.59. For each marker, the maximum PIC value was observed in marker SSR-5 i.e. 0.86 and the minimum was in SSR-11 i.e. 0.41. In a null allele frequencies analysis, all microsatellites had null allele frequencies close to zero. From Table 4.3 it can be concluded that the primer SSR-5 showed 100 % polymorphism percent and also 0.86 PIC value.

Somewhat similar results were put forth by Nematollahi *et al*. (2013) [9] who studied genetic variation among 56 accessions (G1~G56) of *Citrus* including several undefined local or

native genotypes and some known varieties in Jiroft, Kerman province, Iran which were investigated using SSR markers. In total, 12 SSR primers produced 54 alleles. The lowest number of alleles were observed on the cAGG9 locus with 2 alleles and the highest number of alleles were observed on the TAA41 locus with 8 alleles. Polymorphic Information Content (PIC) varied from 0.19 to 0.37 with a mean of 0.28.
Similar results were given by Barkley *et al*. (2006) [2]. They used twenty-four Simple Sequence Repeat (SSR) markers to detect molecular polymorphisms among 37 mostly sexually derived citrus accessions from the collection of citrus germplasm maintained at the University of California. A total of 275 alleles were detected with an average of 11.5 alleles per locus and an average polymorphism information content of 0.625.
SSR-1 produced a total of 13 amplicons of which 11 were polymorphic while 2 were monomorphic. Percent polymorphism had a value of about 85 percent, while the fragment size of amplicons 300 bp (Plate 1).
The fragment size of amplicons 170 bp for SSR-2 with a percent polymorphism value of 87 percent. About 16 amplicons were generated in this primer among which 14 were polymorphic while the remaining 2 were monomorphic (Plate 2).

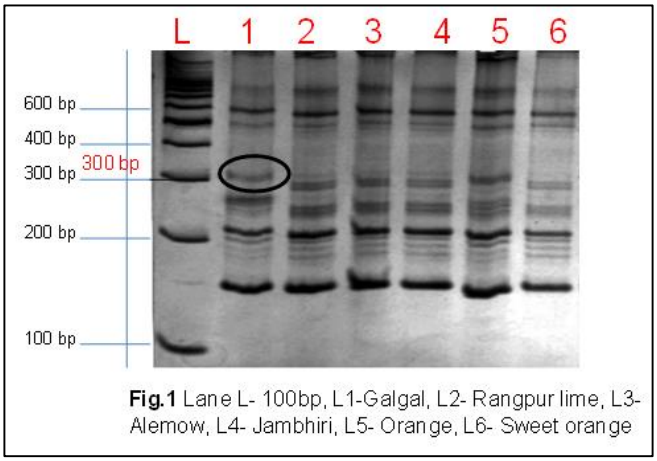

Fig.1 Lane L- 100bp, L1-Galgal, L2- Rangpur lime, L3-Alemow, L4- Jambhiri, L5- Orange, L6- Sweet orange

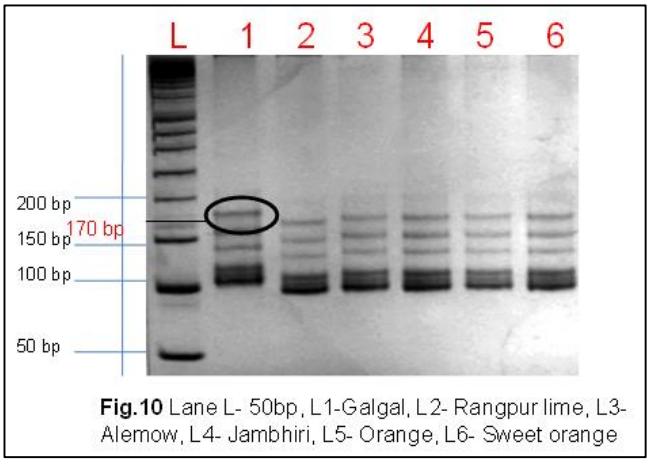

Fig.10 Lane L- 50bp, L1-Galgal, L2- Rangpur lime, L3-Alemow, L4- Jambhiri, L5- Orange, L6- Sweet orange

Plate 1: Six Species of citrus amplified with SSR-1 and SSR-2

A total of 18 amplicons were generated by SSR-3 out of which 12 were polymorphic with a percent polymorphism of 67 percent. The fragment size of amplicons produced 245 bp.
A total of 6 amplicons were generated by SSR-5 out of which all six were polymorphic. The fragment size of amplicons 473 bp while the percent polymorphism was the highest of about 100 percent.

Genetic diversity analysis
SSR markers were used to analyze the genetic diversity of citrus species. The amplicons were then scored using a 1/0 (presence/ absence) system. The similarity coefficient gives the extent of similarity between two genotypes. A lower similarity coefficient value indicates high diversity among the genotypes. The similarity coefficient of genotypes under study is mentioned in Table 2. The highest similarity was found between Galgal and Alemow with a correlation coefficient value of 0.64. The lowest similarity coefficient was observed between Galgal and Jambhiri with a similarity coefficient value of 0.44.
UPGMA Dendrogram was constructed using Jaccard's similarity coefficient which is mentioned in (Figure 1). The Dendrogram showed a clear-cut classification of species into different clusters. The cluster tree analysis showed that the genotypes were broadly divided into two main groups 'A' and 'B' with a genetic similarity value reached 0.50. A cluster including two species was Galgal, Alemow and Rangpur lime; the 'B' group was divided into two sub-clusters; 'B1' and 'B2' with a genetic similarity value of 0.55. The first sub-cluster (B1) included only one species i.e. Jambhiri. A maximum similarity value of 0.64 was observed between two species Alemow and Galgal. The second sub-cluster (B2) included two species Orange and Sweet orange.

Table 2: Jaccard's similarity Coefficient matrix based on SSR markers

Genotypes	Galgal	Rangpur lime	Alemow	Jambhiri	Orange	Sweet orange
Galgal	1					
Rangpur lime	0.557	1				
Alemow	0.645	0.560	1			
Jambhiri	0.440	0.527	0.464	1		
Orange	0.500	0.537	0.557	0.581	1	
Sweet orange	0.518	0.446	0.519	0.517	0.584	1

Similar results were given by Singh *et al*. (2016) [13]. They studied 19 indigenous and exotic mandarin genotypes from different parts of India and USA. Genetic variability and fingerprint profiles were determined using 60 SSR markers. Of the 57 SSR markers amplified, a total of 96 alleles were detected by 39 polymorphic SSR loci and maximum 5 alleles were amplified with an average of 2.46 alleles per primer pair. The CAT01 was the highly informative marker as it revealed a maximum number of alleles (5), PIC value (0.75) and genetic diversity (0.79). Twenty-six SSRs revealed specific/unique alleles and identified nine genotypes including all the hybrids. Across the genotypes, the maximum number of alleles (83) were detected in Daisy hybrid and the percentage of the polymorphic marker was maximum (80.32)

in Nova hybrid. The markers with a low number of alleles were able to differentiate the varieties with specific alleles. The genotypes were classified into three clusters i.e. cluster-I, cluster-II and cluster-III. All the indigenous genotypes were grouped in the cluster -I and it had a maximum genetic similarity coefficient.

Thus, from the present studies, it is concluded that Molecular characterization of six species showed that SSR-1, SSR-2, SSR-3 and SSR-5 are potential markers for discrimination of Galgal and Alemow from other species under study. Similarly, dendrogram constructed on Jaccard's similarity coefficient showed that Galgal and Alemow are more diverse from other species.

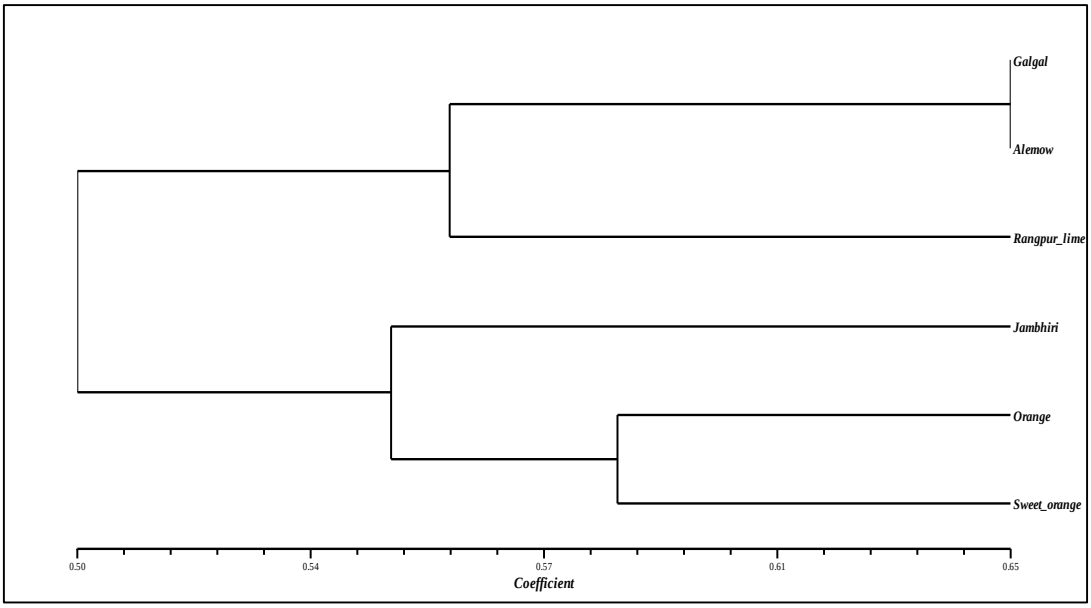

Fig 1: UPGMA dendrogram of six citrus species based on the Jaccard's similarity coefficient using SSR primers**.**

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