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Genetic diversity and DNA fingerprinting of different citrus species using scar markers

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Abstract
Genetic variability and fingerprint profiles of six citrus species were determined using different SCAR markers. In the present investigation, eleven different SCAR markers were validated for discriminating Galgal rootstock from other citrus species under study. The DNA amplification was carried out with eleven SCAR markers. The primer-1, primer-2, primer-3, primer-4, primer-5, primer-6 and primer-11 show the band size of 283 bp, 380 bp, 172 bp, 310 bp, 150 bp, 137 bp and 300 bp respectively. All these primers are promising primers for discrimination of Galgal from other species under study. Two SCAR primers *viz*, primer-1 and primer-6 showed 100% polymorphism followed by primer-2 and primer-3 showed 88% polymorphism with average polymorphism of all primers i.e. 75.90%. Total alleles per locus were 8.27, whereas the average no of monomorphic and polymorphic alleles was 1.63 and 6.54 respectively. The extent of polymorphic information content (PIC) values of eleven SCAR primers ranged from 0.39 to 0.86 with an average value of 0.63. The highest PIC value was observed in primer-1 (0.86), followed by primer-2 (0.76). The minimum PIC value was in primer-10 (0.39). All these primers are promising primers for discrimination of Galgal from other species under study. Out of these primers primer-1, primer-2, primer-6 and primer-11 are the best primers for discrimination of Galgal from Rangpur lime, Alemow, Jambhiri, Orange and Sweet Orange. The highest similarity was found between Jambhiri and Alemow with a correlation coefficient value of 0.48. The lowest similarity coefficient was observed between Orange and Galgal with a similarity coefficient value of 0.22. The cluster analysis revealed that Galgal rootstock is more diverse from other five species under study. These results will be very useful in testing the genetic purity of citrus at nursery stage.

Keywords: Galgal, rootstock identification, SCAR, molecular characterization

1. Introduction
The important commercial citrus fruits grown in India are Mandarin orange (*Citrus reticulata* Blanco), Sweet orange (*Citrus sinensis* Osbeck), Lime (*Citrus aurantifolia* Swingle) and Lemon (*Citrus limon* L.). These are grown commercially in tropical, subtropical, arid, irrigated and mountainous regions in varying soil and weather conditions. Although the citrus industry in India has faced many challenges there is a consistent increase in area and production owing to awareness for sustained production (Kahn *et al*. 2001) [10].

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 fruits are notable for their fragrance, partly due to flavonoids and limonoids contained in the rind, and most are juice-laden. The juice contains a high quantity of citric acids giving them their characteristic sharp flavor. The genus is commercially important as many species are cultivated for their fruit, which is eaten fresh, pressed for juice, or preserved in marmalades and pickles. They are also good sources of vitamin C and flavonoids.

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 (Gaikwad *et al*. 2013) [5].

A variety of methods have been used for citrus cultivar identification. (Luro *et al*. 1995) [11]. 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) [13]. 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) [3].

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

2. Materials and methods

2.1 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.

Species included in the present investigation are,

1. Galgal (*Citrus pseudolimon*)
2. Rangpur lime (*Citrus limonia*)
3. Alemow (*Citrus macrophylla*)
4. Jambhiri (*Citrus jambhiri*)
5. Orange (*Citrus reticulata*)
6. Sweet Orange (*Citrus sinensis*)

2.2 Primer Designing

The species specific primers were developed from the previous sequencing results of amplified product of SCAR. Sequencing was done in bidirectional pattern from Eurofin Genomics Pvt. Ltd. Bengaluru, primer designing was carried out by using Primer Design software of NCBI BLAST.

2.3 Primer design criteria

The following setting was used together with the Primer design tool of NCBI BLAST. Maximum tm difference 2ºC, minimum GC content 40% and maximum GC content 55%, maximum complementarity 2 bp and maximum 32 complementarity 2 bp. Optimum Primer size 20 bp and maximum primer size 23 bp. Optimum Tm 65-68ºC in all the other entries default values were used.

2.4 Primer production - oligo synthesis

The designed primers were synthesized from Sigma Aldrich Bengaluru.

2.5 DNA isolation

Genomic DNA of six citrus species were extracted from young leaves using CTAB method as described by Cheng *et al*. (2003) with minor modifications. The extraction buffer contained 100 mM 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 °C until use. Concentration, quality and quantity of DNA were determined by nano drop spectrophotometrically at 260 nm wavelength.

2.6 PCR amplification

Eleven species specific SCAR 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 Thermo cycler 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. A 50-bp and 100-bp DNA ladder was used to measure the fragment size.

2.7 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 genetic associations between different species were evaluated by calculating the Jaccard's similarity coefficient based on proportion of shared bands produced by primers. The UPGMA Dendrogram was constructed using Jaccard's similarity coefficient.

3. Results

3.1 Molecular characterization of citrus species under study, using SCAR markers

In the present investigation, eleven different SCAR markers were validated for discriminating Galgal rootstock from other citrus species under study. All the eleven primers are promising for discrimination of Galgal from other species under study. A total of 91 amplicons were amplified by 11 polymorphic SCAR loci and the number of amplicons ranged from 4 to 12 with an average of 8.27 amplicons per locus (Table 1). The primer-1, primer-2, primer-3, primer-4, primer-5, primer-6 and primer-11 show the band size of 283 bp, 380 bp, 172 bp, 310 bp, 150 bp, 137 bp and 300 bp respectively. All these primers are promising primers for discrimination of Galgal from other species under study. Out of these primers Primer-1, primer-2, primer-6 and primer-11 are the best primers for discrimination of Galgal from Rangpur lime, Alemow, Jambhiri, Orange and Sweet Orange. Also, primer-1 was found useful for discriminating Alemow species from other five species like Galgal, Rangpur lime, Jambhiri, Orange and Sweet orange at an amplicon size of 510 bp. Also, the band size of 90 bp was identified as a discriminating polymorphic region for Orange species, so it discriminates Orange from other five citrus species *viz.*, Galgal, Rangpur lime, Alemow, Jambhiri and Sweet orange. (Plate 1).

3.2 Unique SCAR amplicons generated in six citrus rootstock genotypes

Seven SCAR primers were found to have a higher discriminating potential for differentiation of the genotypes as they uncovered 13 unique amplicons in six genotypes (Table 2). These primers amplified more than one amplicon. However, one amplicon was amplified in few genotypes that differentiate these genotypes from other citrus rootstock groups. A maximum number of seven unique amplicons was identified in genotypes Galgal followed by two unique

amplicons in Orange. One primer *viz*., Primer-1 revealed unique amplicons in both Galgal at (283 bp) as well as in Orange at (100 bp). But, both the genotypes were differentiated from each other using Primer-2, Primer-3, Primer-5, Primer-6, Primer-7 and Primer-11 markers as these markers revealed unique alleles in Galgal. Primer-7 produced three unique amplicons in Galgal (160 bp), Rangpur lime (155 bp) and Alemow (275 bp), respectively. Two unique amplicons were seen in primer-5 one for Galgal (150 bp) and one in Orange (195 bp). Similarly, Primer-11 generated two unique amplicons, one for Galgal (300 bp) and one in Jambhiri (290 bp).

3.3 Polymorphic information content and percent polymorphism

Two SCAR primers *viz*, primer-1 and primer-6 showed 100% polymorphism followed by primer-2 and primer-3 showed 88% polymorphism with average polymorphism of all primers i.e. 75.90%. Total alleles per locus were 8.27, whereas the average no of monomorphic and polymorphic alleles was 1.63 and 6.54 respectively. The highest PIC value was observed in primer-1 (0.86), followed by primer-2 (0.76) and primer-11 (0.76). The Minimum was in primer-10 (0.39).

3.4 Genetic diversity analysis

SCAR markers were used to analyze the genetic diversity of citrus species. The amplicons were then scored using a 1/0 (presence/ absence) system. The Jaccard's similarity coefficient gives the extent of similarity between two genotypes. A lower similarity coefficient values indicates high diversity among genotypes. The highest similarity was found between Alemow and jambhiri with a correlation coefficient value of 0.48. The lowest similarity coefficient was observed between Galgal and Orange with a similarity coefficient value of 0.22 (Table 3).

The dendrogram constructed on the basis of molecular data. The cluster tree analysis showed that the genotypes were broadly divided into two main groups 'A' and 'B' with a genetic similarity value reached 0.27. A group including individual one species was Galgal; the 'B' group was divided into two sub-clusters; 'B1' and 'B2' with a genetic similarity value of 0.42. The first sub-cluster (B1) included three species *i.e*. Alemow, Jambhiri and Sweet orange. A maximum similarity value of 0.48 was observed between two species Alemow and Jambhiri. The second sub-cluster (B2) included two species i.e. Rangpur lime and Orange (Figure 3).

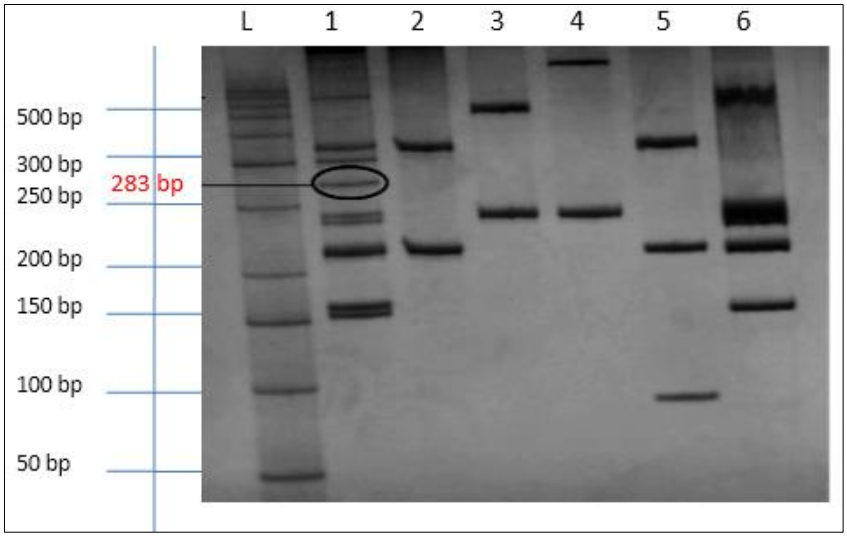

Fig 1: Lane L- 50bp, L1-Galgal, L2- Rangpur lime, L3- Alemow, L4- Jambhiri, L5- Orange, L6- Sweet orange

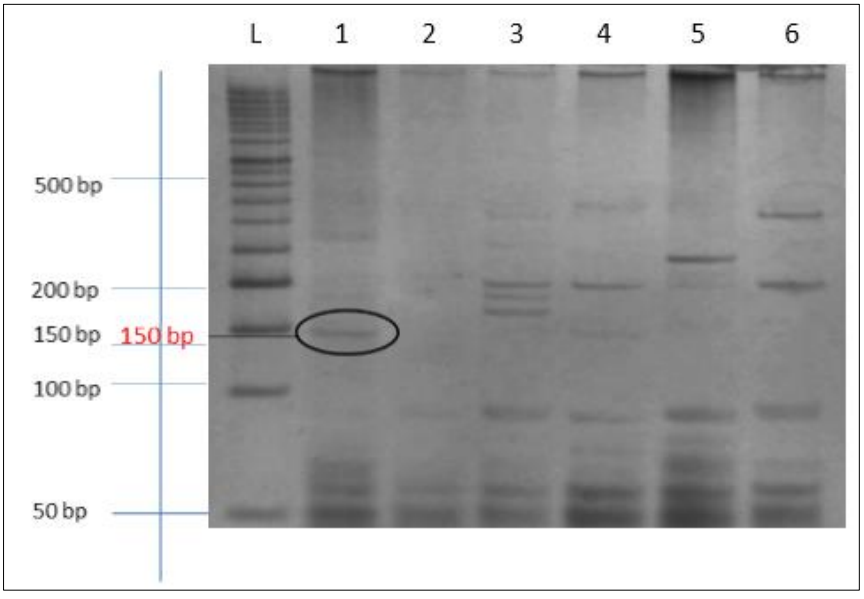

Plate 1: Six species of citrus amplified with primer-1 and primer-5.

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

Sr. No	SCAR primers	No. of amplicons	Monomorphic bands	Polymorphic bands	Polymorphism (%)	PIC value
1.	Primer 1	12	00	12	100	0.86
2.	Primer 2	08	01	07	88	0.76
3.	Primer 3	09	01	08	88	0.70
4.	Primer 4	12	02	10	83	0.68
5.	Primer 5	10	02	08	80	0.60
6.	Primer 6	08	00	08	100	0.72
7.	Primer 7	12	04	08	67	0.66
8.	Primer 8	04	02	02	50	0.47
9.	Primer 9	07	01	06	85	0.70
10.	Primer 10	05	03	02	40	0.39
11.	Primer 11	12	03	08	67	0.76
	Total	91	18	72	835	
	Average	8.27	1.63	6.54	75.90	0.63

Table 2: Specific fragments detected by SCAR primers and distinguished citrus rootstock genotypes

Sr. No.	SCAR Primers	Total number of fragments	Specific fragments	Fragment size (bp)	Genotypes
1.	Primer-1	12	2	283	Galgal
				100	Orange
2.	Primer-2	8	1	380	Galgal
3.	Primer-3	9	1	172	Galgal
4.	Primer-5	10	2	150	Galgal
				195	Orange
5.	Primer-6	8	2	137	Galgal
				130	Sweet orange
6.	Primer-7	8	3	160	Galgal
				155	Rangpur lime
				275	Alemow
7.	Primer-11	12	2	300	Galgal
				290	Jambhiri

Table 3: Jaccard's similarity Coefficient matrix based on SCAR markers

Genotypes	Galgal	Rangpur lime	Alemow	Jambhiri	Orange	Sweet orange
Galgal	1					
Rangpur lime	0.292	1				
Alemow	0.239	0.428	1			
Jambhiri	0.272	0.423	0.482	1		
Orange	0.229	0.461	0.375	0.464	1	
Sweet orange	0.312	0.375	0.428	0.468	0.411	1

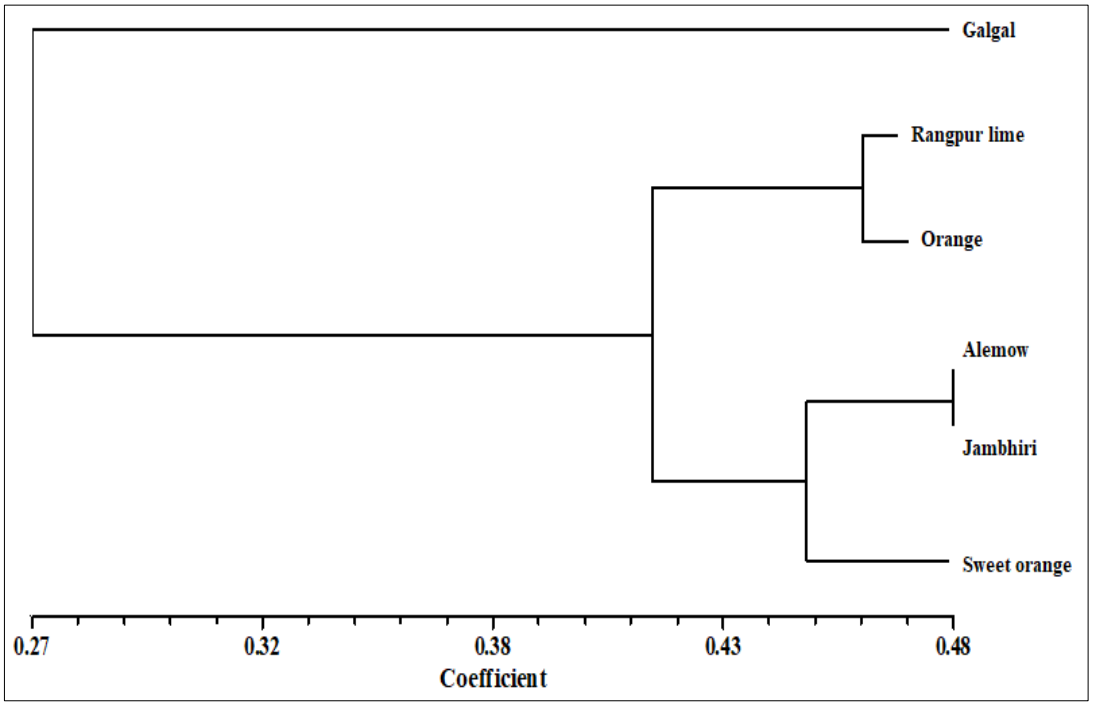

Fig 3: UPGMA dendrogram of six citrus species based on the Jaccard's similarity coefficient using SCAR primers.

4. Discussion

The analysis of rootstock samples with molecular markers proved that SCAR markers were very useful and informative in the differentiation and estimation of genetic diversity within and between the different rootstocks collected from the All India Co-ordinate Research Project on Citrus, Dr. P.D.K.V. Akola and Central Citrus Research Institute, Nagpur. These results are in accordance with other studies using molecular markers to differentiate the different species of citrus (Gaikwad *et al.* 2013; Hvarleva *et al.* 2016) [5, 9]. PCR amplification of the genomic DNA isolated from six citrus species yielded a total of 91 amplicons were amplified by 11 polymorphic SCAR loci and the number of amplicons ranged from 4 to 12 with an average of 8.27 amplicons per locus. These results are quite similar with that of 7-15 fragments ranging per primer and with an average 10.8 fragments, reported by Hussein *et al.* (2003) [8] among different citrus accessions.

The extent of polymorphic information content (PIC) of eleven primers ranged from 0.39 to 0.86. Comparable outcome was shared by Romdhane *et al.* (2016) [14] and Barkley *et al.* (2006) [2] where they found PIC values ranged from 0.5-0.7 with an average value of 0.625 among different citrus accessions. Specific alleles were identified with SCAR markers, they were able to differentiate rootstocks. The primer-1, primer-2, primer-3, primer-4, primer-5, primer-6 and primer-11 show the band size of 283 bp, 380 bp, 172 bp, 310 bp, 150 bp, 137 bp and 300 bp respectively. All these primers are promising primers for discrimination of Galgal from other species under study. Out of these primers Primer-1, primer-2, primer-6 and primer-11 are the best primers for discrimination of Galgal from Rangpur lime, Alemow, Jambhiri, Orange and Sweet Orange. Also, primer-1 was found useful for discriminating Alemow species from other five species like Galgal, Rangpur lime, Jambhiri, Orange and Sweet orange at an amplicon size of 510 bp. Also, the band size of 90 bp was identified as a discriminating polymorphic region for Orange species, so it discriminates Orange from other five citrus species *viz.*, Galgal, Rangpur lime, Alemow, Jambhiri and Sweet orange. The foregoing outcome is matching with the work specified by Gaikwad *et al.* (2018) [6] where they used the Sequence Tagged Microsatellite marker for identification of three citrus rootstocks namely; Galgal, Jambhiri and Rangpur lime. They found that an allele of 160 bp was amplified in all the three genotypes. However, in Jambhiri and Rangpur lime, an additional allele of 200 bp and 180 bp, respectively was present. So, on the basis of presence/absence of fragment, Jambhiri and Rangpur lime can be differentiated from Galgal. These primers are very useful in a breeding program since they can help to follow unique fragments in the generations and could be used as marker-assisted selection.

The clustering based on UPGMA analysis revealed the genetic variation and relationship among different species. The dendrogram showed clear cut classification of species into two different clusters. We could notice from the dendrogram, that Galgal rootstock form a separate group. The highest similarity was found between Alemow and jambhiri with a correlation coefficient value of 0.48. The lowest similarity coefficient was observed between Galgal and Orange with a similarity coefficient value of 0.22. Similar results were obtained by Uchoi (2017) [15] wherein they indicated that the 12 citrus germplasms were grouped into two major clusters likewise Hamza (2013) [7] and Malik *et al.* (2013) [12].

5. Conclusion

In the present study, Molecular characterization of six species showed that primer-1, primer-2, primer-6, primer-7 and primer-11 are potential markers for discrimination of Galgal from other species under study. Similarly, dendrogram constructed on Jaccard's similarity coefficient showed that Galgal is more diverse from other species. The genotypes categorized in different clusters can be used by breeder to develop new cultivars. Therefore, the set of SCAR markers used in present study were successful in fingerprinting and evaluating genetic diversity in the citrus species which will be of great utility for breeding of citrus germplasm. These results will be very useful in testing the genetic purity of citrus seedling at nursery stage.

6. Conflict of interest

The authors declare that there are no conflicts of interest relevant to this article.

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