Seed Research 47(1): 15-28, June, 2019

Genetic Purity Test using SSR Markers in Pigeon Pea

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(Received: April 2019; Revised: May 2019; Accepted: June 2019)

ABSTRACT: Pigeon pea is one of the strategic and vital dry land crops having significance in food grain sustainability and nutrition. The seed testing especially genetic purity assumes greater importance in quality seed availability to the farmers. The present investigation aims at developing faster method using molecular methods using simple sequence repeat (SSR) markers for ten varieties of pigeon pea (Maruti, TS3R, GRG-811, Asha, BSMR-736, GRG-833, GC-11-39, WRP-1, ICPL-87 and Bannur local). Forty three SSR markers were used to distinguish ten pigeon pea varieties with average 2.81 alleles per marker. Out of which sixteen markers were polymorphic to these ten varieties having PIC values ranging from 0.567 to 0.695. The variety specific markers *viz.,*CcM0039 (GC-11-39), CcM0047 (GRG-833), CcM0057 (Maruti), CcM0082 (GRG-811), CcM0093 (Bannur Local), CcM0095 (GC-11-39), CcM0133 (Maruti), CcM0185 (WRP-1), CcM0193 (GRG-833), CcM0248 (WRP-1), CcM0252 (ICPL-87), CcM0257 (ICPL-87), CcM0271 (BSMR-736), CcM0293 (TS3-R), AHSSR46 and AHSSR48 (Asha) were used for conducting molecular genetic purity tests. The cluster analysis of all the ten pigeon pea varieties based on the morphological characters and SSR markers revealed differences in the cluster pattern. There were only two clusters based on the morphological scoring of characters but three clusters using SSR markers by fixed Dice similarity coefficient of 3.71. These tests were further validated by field grow out test. The correlations of field grow out test and molecular genetic purity by paired t-test with calculated t-value (1.66) less than the table value (2.26) showing no significant differences between these two methods. Also there is positive correlation exist between these two methods (r = 0.77).

Keywords: Pigeon pea, Molecular genetic purity test, SSR markers, PIC, Cluster analysis, Field grow out test

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is a most valuable cash crop in semi-arid tropical regions due to its tolerance to drought [1] and enriches the soil with nitrogen fixation [2]. Apart from this, it can store well when dried and hence can provide year-round nutritional security. Also, it helps in the alleviation of poverty because of its diverse uses as food, fodder and fuel [3]. Cultivated pigeon pea is an excellent source of protein (20-22 %), dietary fibre, and various vitamins: magnesium, phosphorus, potassium, copper, and manganese. Though hybrid technology in pigeon pea is on the verge of commercialisation in India, varieties are still more popular in pigeon pea growing areas. Supply of quality seed of these varieties which are genetically pure is very critical to ensure their potential for which they are being developed. Since pigeon pea is often cross-pollinated (3-45 %), there is a possibility of genetic contamination during its seed production cycle [4].

The seed produced need to be tested in field grow-out-test (GoT) for genetic purity before it is released for commercial cultivation thus confirming its genuineness and true-to-type characters. Field grow-out test to determine genetic purity in pigeon pea relies on morphological characters which will be evaluated at different phases of crop growth. Most of the popular varieties cultivated in the southern part of India are having similar morphological characters except for flower characters. Further, the improved varieties of pigeon pea grown in the southern part of India are medium to long durational having maturity period ranging from 150 to 180 days. Thus to find out genuineness of variety through field GoT, the crop has to be maintained till flowering which is up to a minimum of 130 days in field GoT.

Keeping the crop for such a very long time without declaring results of genetic purity may delay the seed distribution for commercial cultivation and grower may miss the recommended sowing window. This may also add expenses of storage and handling to the final value of the seed. Hence, conducting field GoT in pigeon pea is limited due to time constraints and dependent on environment [5] while, identification of simple sequence repeats (SSR) markers to assess genetic purity can speed up the seed testing and results can be available within shortperiod [6].

MATERIALS AND METHODS

Molecular Characterisation of Pigeon pea Varieties using SSR Markers

The ten varieties of pigeon pea seeds grown during two seasons for morphological characterisation using distinctness, uniformity and stability (DUS) criteria are used for molecular characterisation. The varieties viz., Maruti, TS3R, GRG-811, Asha, BSMR-736, GRG-833, GC-11-39, WRP-1, ICPL-87 and Bannur local were used. Genetically pure seeds or true to type as described by the breeder were collected from plants marked for collection during morphological characterisation from the crop grown at ARS, Annigeri during 2017 and 2018 (two seasons). All the molecular tests were conducted in Seed Biotechnological Laboratory at Department of Seed Science and Technology, UAS, Dharwad.

Plant Material

Seeds of pigeon pea varieties were planted in a raised bed nursery of 2.5 m. Seedlings were grown for 12-15 days and 20 g of leaf samples were collected from these seedlings. Approximately, 2 mm^2 Leaf was harvested into a sterile 1.7 ml Eppendorf tubes and stored under -20^0C temperature until the extraction of genomic DNA. Leaf samples in Eppendorf tubes were stored in aluminium foils.

Molecular Analysis

DNA extraction

The genomic DNA was extracted using the CTAB method from the top most fully expanded leaf samples of two-week-old plants for each variety following the general protocol [7-8].

1. Add CTAB and Grind the leaf tissue (10 min)
2. Incubate at 60^oC (60 min)
3. Chloroform extraction and transfer the supernatant (60 min)
4. DNA precipitation at 2000 rpm and Incubate at 60^oC (30 min)
5. Ethanol wash (10 min) and Incubate at 60^oC (30 min)
6. DNA clean up (30 min)

Quality of the DNA

The quality of DNA can be assessed by spectrophotometer and fluorometry methods. The quantity of DNA can be measured in microgram and nanogram (one microgram is 1/6^{th} of a gram and one nanogram is 1/9^{th} of a gram). The DNA which is visible in the wavelength of 260-280 nm is of good quality which absorb UV light only and if <260 nm or >320 nm can be of inferior quality due to the presence of protein. This DNA absorbs all type of light including UV.

i. *Visualization:* DNA banding pattern can be visualised by electrophoreses which is helpful in distinguishing different DNAs in contaminants and pure seeds. Though the gel electrophoreses give distinct banding pattern but to have clear distinction PCR was used to amplify the DNA. Here the DNA from pigeon pea Cv TS3R and other contaminants were run with different SSR markers.

ii. *PCR Programme and analysis:* PCR reaction performed in 50 µl final volumes with mixture of 25 ng of genomic DNA, 5 µl 10X PCR buffer, 0.5 mM mix dNTP, 1.5 mM MgCl2, 0.5 mM of each forward and reverse primers and 0.2 U of *Taq*DNA polymerase. The amplification figure consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 30 seconds at the annealing temperature, 1 minute for elongation at 72°C, and a final extension step of 5 minutes at 72°C [9].

Quantification of Genomic DNA

The presence and quality of DNA were evaluated by electrophoresis on 1% agarose gel at 100 V for 120 min in 1X TAE (Tris-base, glacial acetic acid, EDTA) gel buffer. The gel was stained with 0.25 µg/ml ethidium bromide for DNA visualization [10].

List of Markers used in the Study

There were 43 SSR markers used for the present study are presented in table 1. The preparation of these SSR markers as per the F/R sequences were custom procured from Azyme Biosciences, Bangalore. These markers were collected from the public domain and ICRISAT repository [11].

SSR Marker Screening

Screening for polymorphic primers for each variety was studied. A total of 43 SSR markers were subjected for screening, to obtain a single and distinct polymorphic primer for each variety of pigeon pea under study. The DNA extracted from plants of each varietywere subjected for primer screening. The stringency of the PCR conditions was varied to maximum to overrule the possibility of non-specific amplification. The polymorphic primers were selected, PCR conditions were optimizedand the DNA of 93 plants from each variety of a crop was subjected for PCR amplification along with their respective parental lines. A negative control with no DNA was used during PCR amplification.

Grow Out Test for Validity of Molecular Test Results

A field grows out test was conducted on all the varieties under study as per ISTA procedure [12] to validate the results of the moleculartest in the GoT plot of UAS, Raichur. All the agronomic practices and plant protection measures were implemented as per recommended practices for raising healthy crops. In the Grow-out field trials, genetic purity evaluation was conducted based on morphological characters. The characters of few individuals that showed variation from the standard morphological traits were recognized as off-type and they were compared to those of the parental seeds showing 97 per cent purity, which was also supported by the molecular marker testing.

Table 1. Polymorphic Information Content (PIC) of the SSR markers used in the study

Sl. No	Primer	Forward (F) / Reverse (R) Primer sequence 5'-3	Ann. Temp. (Tm)	Size-bp	No. of alleles	PIC
1	CcM0008F	CGGTGAAAAGGGTCAATGAG	46.8	182	3	0.581
	CcM0008R	CAAAATTAAAGCCTACTTATTTTACGA				
2	CcM0021F	TGAATGTTTTCCAGGATTTTACA	46.8	280	2	0.364
	CcM0021R	GCGCAAATATAAGAGCCCAG				
3	CcM0030F	GCAATATCAATTCAATGGTGGA	48.0	218	2	0.375
	CcM0030R	TGACAGATGCACTCTCTCGTTT				
4	CcM0039F	AGGAATAATGTTTGCTGCGG	44.7	261	3	0.592
	CcM0039R	TTGGTATGTGGAACGATTGC				
5	CcM0047F	TGTCTTTTGGATGAAAGTAGGGA	46.8	160	4	0.587
	CcM0047R	GTTGGGGATGGGAAGAGAAT				
6	CcM0051F	ACCTTTATTTTGAGCAGGAAAA	44.2	269	3	0.592
	CcM0051R	TGAATCATTTTCTGTTGAAGGG				
7	CcM0057F	CAATGTTGGCATAGGAACCA	44.7	269	3	0.567
	CcM0057R	GCTTAAAACTTGTGGGGCAA				
8	CcM0080F	TGCATGTTGTATGTTGGTTGG	45.5	173	2	0.304
	CcM0080R	GTAATGCATCTCAATAATTTCAACA				
9	CcM0082F	TGTCAATGTCATGTTGGGCT	46.8	245	4	0.695
	CcM0082R	CATGCCATCTTCCTTTCTCC				
10	CcM0093F	TCATTGACCCCTCTGGAAAT	44.7	269	4	0.671
	CcM0093R	ACAATTGGAAAAATAAGTGAGTGAT				
11	CcM0095F	ATAATTAGTGGGCTGGGCCT	46.8	208	3	0.581
	CcM0095R	TGCCTCATAATCATGTTGCTTC				
12	CcM0112F	GCAGGCATGCAAGCTTATTA	44.7	148	3	0.535
	CcM0112R	GCGCGCCATCTTAATATCAT				
13	CcM0121F	AGAAATTGGAGGCTTGGTCA	46.8	273	2	0.364
	CcM0121R	GGTATAAGGCTCAAACCCGA				

Sl. No	Primer	Forward (F) / Reverse (R) Primer sequence 5'-3	Ann. Temp. (Tm)	Size-bp	No. of alleles	PIC
14	CcM0126F	TGGTCCATGTTCCTCACTCA	46.8	218	2	0.375
	CcM0126R	CCAATGAAAATGAGAACCTTCA				
15	CcM0133F	GTTGTCCCATTTTGACCTCC	46.8	176	3	0.581
	CcM0133R	CCATAATCCAATCCAAATCCA				
16	CcM0134F	CTCTGCCCCGATGTCATATT	46.8	240	4	0.684
	CcM0134R	TTGGGATGTGAAGATGATGAA				
17	CcM0179F	GCAAAATTGCACTAAAATTTGTTT	46.8	189	2	0.345
	CcM0179R	CCATCTTCGCCTGTCGTATT				
18	CcM0183F	GCCCATTTTGTCATCCCTAA	44.7	236	2	0.364
	CcM0183R	TTCAACAGTTGGATCGTTCA				
19	CcM0185F	TTGATCATGACTTATGCCTTTGA	46.8	232	3	0.595
	CcM0185R	GGCTTGCTTTGAGTTCCTTG				
20	CcM0193F	TAAATCACCACCCTTGAGGC	46.8	190	3	0.567
	CcM0193R	TGCAAAAACACATCCTGGAA				
21	CcM0195F	CAACAATAAAGCATAAACCACCA	48.5	223	3	0.579
	CcM0195R	TGACGTAGATTGGGTAGTTAGGA				
22	CcM0207F	TTTTGGCGGTCATTTTAACC	43.9	235	2	0.364
	CcM0207R	TTAGTCGGGAGCAACATGA				
23	CcM0208F	GCATCTAAATACAATTAATATTGTGGG	48.8	122	3	0.592
	CcM0208R	ATAGGGTGGATCTCTGGTGC				
24	CcM0246F	ATGGAGCCAAAGTGTCCAAG	46.8	226	2	0.364
	CcM0246R	ATGAAAAGCAACTACGCGCT				
25	CcM0248F	TGGAATTTGACTCATTTAGAATAGGA	47.4	279	4	0.579
	CcM0248R	CCCACAGACAGCATATCAACA				
26	CcM0252F	CATAGAAGCCCACCTTCCAA	46.8	234	3	0.579
	CcM0252R	CTGCATGCAAAACGAAGAAG				
27	CcM0257F	GCCGTTACGAGGGTAATGAA	48.8	241	3	0.581
	CcM0257R	CTGTCTCAAAGGGACCCTGA				
28	CcM0268F	CCTTTTGGGTTAGGGTATCCA	48.8	210	2	0.375
	CcM0268R	CCCCTAACGTAGCCTGTCAA				
29	CcM0271F	TGCTTCGCATTCCTCTTTTT	44.7	268	4	0.581
	CcM0271R	AGGAAAATGCTGCTTTGCAC				
30	CcM0273F	CAGGTATACGCTGCTCACCA	48.8	264	3	0.581
	CcM0273R	GCAGTTGCTGATGGAGTTGA				
31	CcM0293F	GTTCCCGGCTACCAAATGTA	46.8	231	4	0.687
	CcM0293R	AAAAACACAAAAGATAAACATACATGC				
32	CcM0303F	CAAGCTTTTGAGGTTCGACA	44.7	205	4	0.693
	CcM0303R	TCACGCAAGAAATTCACAGC				
33	CcM0353F	GATTCGCAAGTTGCTCCTTC	46.8	189	3	0.592
	CcM0353R	TTGTGATCACTTCATCATTTTGG				
34	CcM0361F	TCTTCCTGTCCTCATCCTCG	48.8	172	2	0.364
	CcM0361R	TGGAAACCAAAGTTGTGCAT				

Sl. No	Primer	Forward (F) / Reverse (R) Primer sequence 5'-3	Ann. Temp. (Tm)	Size-bp	No. of alleles	PIC
35	CcM0366F	CCTATGCTCCACAACAAGAAAA	46.7	170	2	0.375
	CcM0366R	GCGATAGAAACATATTGCACACA				
36	CZ681920F	GCGGGATTCTCTTGCTTAC	46.1	205	2	0.364
	CZ681920R	TCACAAAACAATTTGGCACA				
37	CZ681922F	ACACCACCATGCTAAAGAACAAG	49.0	221	3	0.579
	CZ681922R	CCAAGCAAGACACGAGTAATCATA				
38	CZ681923F	CATCGCCTACAATCATACAAAGA	46.7	170	2	0.375
	CZ681923R	TCTTGTCCTTTTTCAGTCATCGT				
39	CZ681924F	ATCGCTTTGCATCCTTATC	41.8	194	4	0.693
	CZ681924R	CTTCACGTACATTTTCGTTT				
40	AHSSR38 F	CACGTATAACAAATTGAAACCA	47.6	185	3	0.579
	AHSSR38 R	GATGTGAGACAACGAGATGTT				
41	AHSSR44 F	ACTGAACCCAATAATGTTTGA	48.2	211	2	0.375
	AHSSR44 R	AAATGGGGGTACTCGTATTAG				
42	AHSSR46 F	CATCGAGGTTGTTATTTTGTT	49.2	174	4	0.687
	AHSSR46 R	TTTAAGTTGGTCCGTCGATA				
43	AHSSR48 F	TCATGTCTATGCACTTAATCG	46.9	183	3	0.581
	AHSSR48 R	ACCATACCACGACTGTCATAC				

Data Analysis

Marker polymorphism

To measure the informativeness of the markers, the polymorphism information content (PIC) (http://www.agri.huji.ac.il/-Weller/Hayim/parent/PIC) for each SSR marker was calculated according to the formula:

$PIC = 1-\Sigma\Sigma Pi^2 - \Sigma\Sigma Pi^2\, Pj^2$

Whereas, 'i' is the total number of allele detected for SSR marker

'Pi' is the frequency of the 'i'th allele in the set of 10 genotypes investigated and j = i+1.

This formula gives us an indicator of how many alleles a certain marker has, and how much these alleles divide evenly.

Calculation of genetic purity using the molecular method

To assess the off-types percentage, the amplified PCR product of the parental line and samples of each variety were resolved by electrophoresis in 1.8 per cent agarose gel. The banding pattern of variety was assessed corresponding to the parental line and inference was drawn for an off-types percentage. This result was validated with the results of grow-out test results

Cluster Analysis

To analyse whether all the pigeon pea varieties under this investigation are related based on the morphological characters, a scoring based on the morphological characters was done as per the notes (1 to 9) which were used to describe the state of each character for the purpose of digital data processing. The dendrogram constructed using morphological characters were based on the scoring of characters as per the DUS criteria. For dendrogram construction Mahalanobis Squared Euclidian (Euclidian^2) technique was employed using SPSS version-25 (IBM™ Corporation).

To analyse whether all the pigeon pea varieties under this investigation are related based on the molecular methods based on the 43 SSR markers, Unweighted Pair Group Methods with Arithmetic Means (UPGMA) technique was employed using SHAN programme of NTSYS version-2.2 (Exeter ™ Software). The dendrogram constructed using binary data for the presence and absence of banding pattern amplified by the varieties specific to markers [13].

RESULTS AND DISCUSSION

SSR Marker Polymorphic Primer Screening and Marker Polymorphism

In the present investigation a total of 43 SSR markers were used for ten pigeon pea varieties which are either released varieties or local and germplasm in pipeline for variety development. Markers with polymorphism information content (PIC) values of 0.5 or higher are considered as highly informative and useful for distinguishing the polymorphism at specific locus. The PIC data is very important to select specific marker to a particular variety. These observations were in line with earlier results in pigeon pea inter specific crosses by [13] who quantified diversity among 12 genotypes comprising six stable CMS lines and their maintainers derived from inter-specific crosses *viz*., *C. scarabaeoides* × *C. cajan* and *C. cajanifolius* × *C. cajan*.

The selected SSR markers showed a high level of polymorphism, as their corresponding polymorphism information content value ranged from 0.58 to 0.72 with an average PIC of 0.68. The DNA based approaches generally detect more polymorphism over other methods and constitute a new generation of genetic markers [5, 6, 14]. Also based on DNA sequence variation these provide an unbiased means of identifying crop varieties. These markers can be employed with medium quality DNA and have high reproducibility with low technical complexity. However, this method involves a high level of automation [15].

Identification of Variety Specific SSR Marker

The identification of variety specific SSR markers was based on the Polymorphic primer screening and PIC values and one prominent polymorphic marker having high PIC value was identified for each of varieties which were used for molecular genetic purity assessment of the random sample of each variety (Figure 1).

The amplified sizes of allele of each of marker for each of varieties were recorded. Among the ten varieties, Maruti can be distinguishable using the SSR marker CcM0057 and CcM0133. TS3R and GRG-811 can be distinguishable using CcM0293 and CcM0082 respectively. Asha can be easily distinguishable by using two SSR markers namely AHSSR46 and AHSSR48.

The SSR markers CcM0271 can distinguish the variety BSMR-736. The variety GRG-833 can be distinguishable clearly by two SSR markers CcM0047 and CcM0193. Similarly the variety GC-11-39 can be distinguishable clearly by two SSR markers CcM0039 and CcM0095. The variety WRP-1 can be distinguishable using CcM0185 and CcM0248.The SSR markers CcM0252 and CcM0257 can distinguish the variety ICPL-87. Whereas, CcM0093 can clearly distinguish the variety Bannur local. These SSR markers were having >0.5 PIC values and can be used as molecular descriptors to the varieties. These observations were in line with [16] who isolated 36 SSR markers, of which 13 markers were polymorphic among 32 cultivated and eight wild pigeon pea genotypes. Several workers have demonstrated similar observations on the remarkable potential of SSR markers to discriminate between pigeon pea genotypes [13, 17-19].

In pigeon pea, SSR markers can be useful tools to assess genetic purity and diversity of varieties [16, 17]. Kim *et al.* [20] developed a technique for cultivar discrimination using SSR markers in soybean. A total of 91 soybean cultivars developed from 1913 to 2002 in Korea Republic was evaluated by five polymorphic SSR markers (Sat-043, Sat- 036, Sat-022, Sat-088 and Sat-045). These SSR markers generated a total of 64 alleles and the number of alleles for each SSR marker ranged from 10 to 15 with an average of 12.8.

Singh *et al.* [18] used 22 SSR markers of different crop species origin to assess polymorphism through their SSR fingerprinting of 16 cultivated pigeon pea genotypes. 425 bands were amplified in all the 16 genotypes and a total of 46 SSR fragments were amplified. They reported that the specific bands developed by the SSR primers could also be used for cultivar identification. Odeny *et al.* [1] succeeded in designing 113 pigeon pea SSR markers, 73 of which had amplified interpretable bands. 35 markers revealed polymorphism among 24 pigeon pea breeding lines.

Saxena *et al.* [16] isolated 36 microsatellite or simple sequence repeat (SSR) loci from an SSR-enriched genomic library and among these 13 SSR markers were polymorphic amongst 32 cultivated and eight wild pigeon pea genotypes representing six *Cajanus* species. Njunge *et al.* [21] identified six markers and screened allelic data associated with five most popular cultivated varieties of pigeon pea in Malawi. Based on this they opined that using this genetic finger printing can be potentially applied for seed certification to confirm the genetic purity of seeds. Sarkar *et al.* [19] revealed that considerable molecular

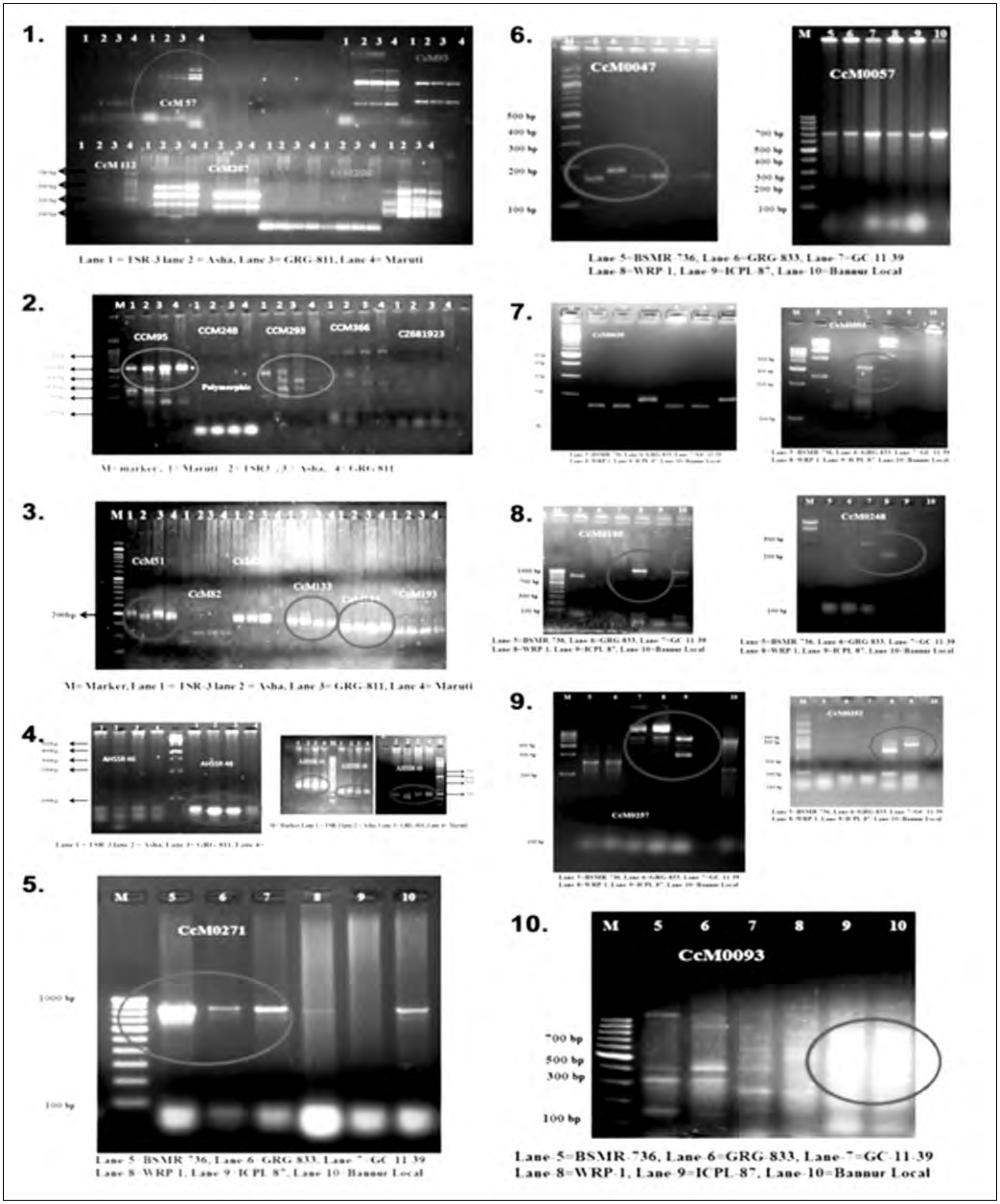

Figure 1. Identification of variety specific SSR marker

1: CcM0057 for Maruti; 2: CcM0293 for TS3R; 3: CcM0082 for GRG-811; 4: AHSSR46 for Asha; 5:CcM0271 for BSMR-736; 6: CcM0047 for GRG-833; 7: CcM0095 for GC-11-39; 8: CcM0185 for WRP-1; 9: CcM0257 for ICPL-87; 10: CcM0093 for Bannur Local genetic diversity among genotypes of pigeon pea using SSR markers. One hundred and thirty-eight pigeon pea genotypes were analyzed for molecular genetic diversity using 34 SSR markers with a putative function for drought tolerance. The study revealed that fifty two alleles were obtained with 34 SSR markers while 1 to 3 alleles were scored with an average of ~1.6 alleles for each SSR. Three alleles were amplified by markers ASSR 1, ASSR 93 and ASSR 97. Of these, 15 SSR markers were found to be polymorphic which identified 33 alleles among 138 genotypes. The average PIC value of these polymorphic SSRs was 0.22 with a range of 0.01 for ASSR 308 to 0.38 in ASSR 97.

The usefulness of molecular markers for either DNA fingerprinting or genetic purity testing is demonstrated as early as 1988 in paddy [22]. The use of variety specific SSR markers can be useful for conducting genetic purity. These observations were in line with other workers to use DNA approaches to test genetic purity [23, 24]. Conducting field GoT in pigeon pea is limited due to time constraints and dependent on environment [5]. While, identification of simple sequence repeats (SSR) markers to assess genetic purity can speed up the seed testing and results can be available within shortperiod [6].

Molecular Genetic Purity Test with Variety Specific SSR Marker

To find genetic purity test using the SSR markers, molecular genetic purity test was done (Table 2). Among the ten varieties, in Maruti, out of 36 seeds tested there were three lapses (Figure 2). Out of 33 seeds one seed at lane 4 was recorded as off-type which was heterozygous with the SSR marker CcM0057 and remaining 32 are homozygous with variety specific allele with molecular genetic purity (MGP) of 96.96 per cent. In TS3R, out of 19 seeds tested there were two lapses and two are off-types at lane 13 and 14 (Figure 3). Remaining 15 are homozygous with variety specific allele of the marker CcM0293. The MGP for TS3R was recorded as 88.23 per cent.

In GRG-811, out of 25 seeds tested there was one lapse. Out of 24 seeds three seeds at lanes 15, 24 and 25 were recorded as off-types which were heterozygous with the SSR marker CcM0082 and remaining 21 are homozygous with variety specific allele with molecular genetic purity (MGP) of 87.50 per cent (Figure 4). In Asha, out of 25 seeds tested there was only one lapse and one was off-type at lane 12. Remaining 23 are homozygous with variety specific allele of the marker AHSSR 46 with MGP of 95.83 per cent (Figure 5).

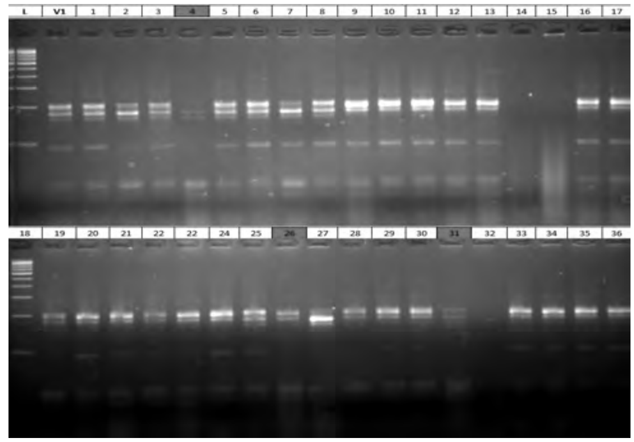

Figure 2. Molecular genetic purity test of V1: Maruti with SSR Marker CcM0057

Legend: Lanes 4, 26 and 31 are off-types; Lanes 14, 15 and 32 are lapses without any banding pattern

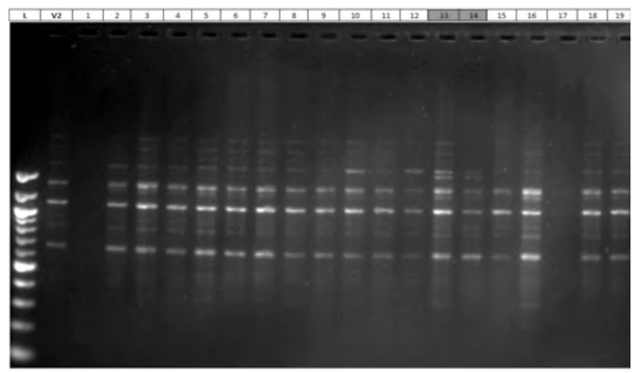

Figure 3. Molecular genetic purity test of V2: TS3R with SSR Marker CcM0293

Legend: Lanes 13 and 14 are off-types; Lanes 1 and 17 are lapses without any banding pattern

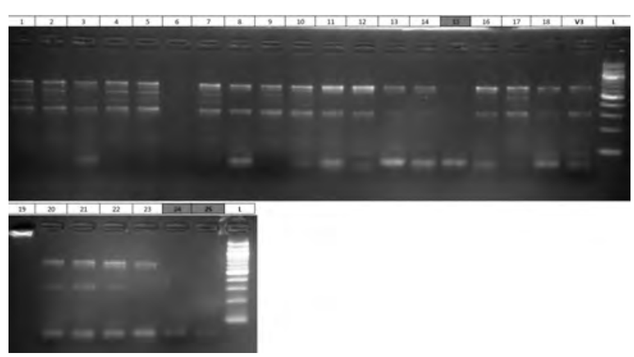

Figure 4. Molecular genetic purity test of V3: GRG-811 with SSR Marker CcM0085

Legend: Lanes 15, 2.4 and 25 are off-types; Lane 6 is a lapse without any banding pattern

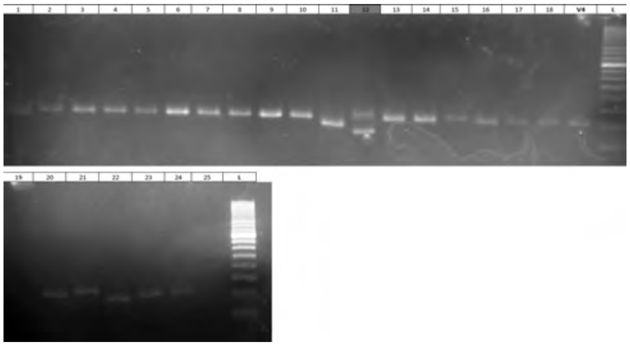

Figure 5. Molecular genetic purity test of V4: Asha with SSR Marker AHSSR-46

Legend: Lane 12 is an off-types; Lane 19 is a lapse without any banding pattern

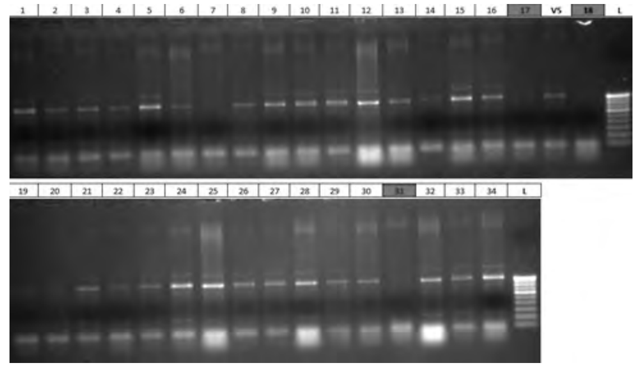

Figure 6. Molecular genetic purity test of V5: BSMR-736 with SSR Marker CcM0271

Legend: Lanes 17, 18 and 31 are off-types; No lapses

Table 2. Molecular genetic purity test with variety specific SSR marker

Sl No	Variety	SSR Marker	Seed Sample (No.) *	Contaminants Added	Total Bands **	Total off-types	Total Lapses in Bands #	Molecular Genetic Purity % ##	Impurity % due to off-types
1	Maruti	CcM0057	36	05	33	01	03	96.96	3.04
2	TS3R	CcM0293	19	02	17	02	02	88.23	11.77
3	GRG-811	CcM0082	25	03	24	03	01	87.50	12.50
4	Asha	AHSSR 46	25	02	24	01	01	95.83	4.17
5	BSMR-736	CcM0271	34	03	34	03	0	91.17	8.83
6	GRG-833	CcM0047	23	03	23	03	0	86.95	13.05
7	GC-11-39	CcM0039	15	02	14	01	01	92.85	7.15
8	WRP-1	CcM0185	25	02	25	02	0	92.00	8.00
9	ICPL-87	CcM0252	36	03	35	02	01	94.28	5.72
10	Bannur Local	CcM0093	15	02	14	02	01	85.71	14.29
							Mean	91.20~	8.80
							Range	85.71-96.96	3.04-14.29
							S.D	3.85	3.85
							C.V (%)	0.042	0.44

* Seed sample included contaminants added

** Total bands obtained for each of seeds in sample

Lapses in obtaining banding pattern using specific SSR marker

Molecular genetic purity percentage obtained calculating the total bands obtained

In BSMR-736, out of 34 seeds tested there was no lapse. Out of 34 seeds three seeds at lanes 17, 18 and 31 were recorded as off-types which were heterozygous with the SSR marker CcM0271 and remaining 31 are homozygous with variety specific allele with molecular genetic purity (MGP) of 91.17 per cent (Figure 6). In GRG-833, out of 23 seeds tested there was no lapse and three were off-types at lanes 10, 17 and 18. Remaining 20 are homozygous with variety specific allele of the marker CcM0047 with MGP of 86.95 per cent (Figure 7).

In GC-11-39, out of 15 seeds tested there was one lapse. Out of 14 seeds one seed at lane 13 was recorded as off-type which was heterozygous with the SSR marker

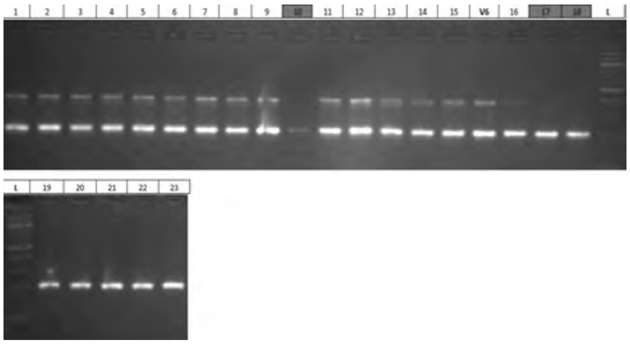

Figure 7. Molecular genetic purity test of V6: GRG-833 with SSR Marker CcM0047

Legend: Lanes 10, 17 and 18 are off-types; No lapses

CcM0039 and remaining 13 are homozygous with variety specific allele with molecular genetic purity (MGP) of 92.85 per cent (Figure 8). In WRP-1, out of 25 seeds tested there was no lapse and two were off-types at lanes 11 and 13. Remaining 23 are homozygous with variety specific allele of the marker CcM0185 with MGP of 92.00 percent (Figure 9).

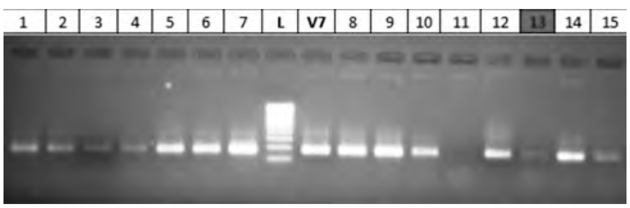

Figure 8. Molecular genetic purity test of V7: GC-11-39 with SSR Marker CcM0039

Legend: Lane 13 is an off-types; Lane 11 is a lapse

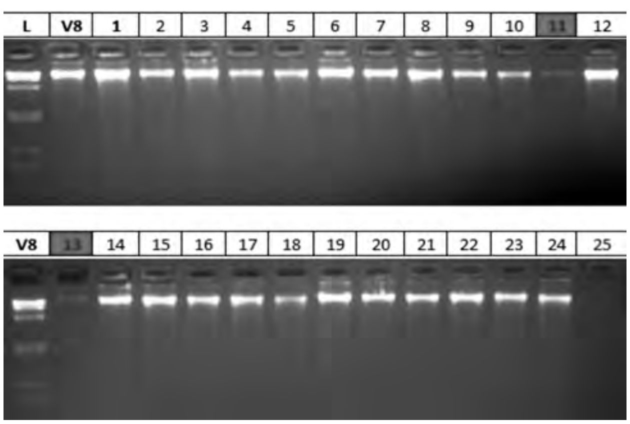

Figure 9. Molecular genetic purity test of V8: WRP-1 with SSR Marker CcM0185

Legend: Lanes 11 and 13 are off-types; Lane 25 is a lapse

In ICPL-87, out of 36 seeds tested there was one lapse. Out of 35 seeds two seeds at lanes 11 and 32 were recorded as off-types which were heterozygous with the

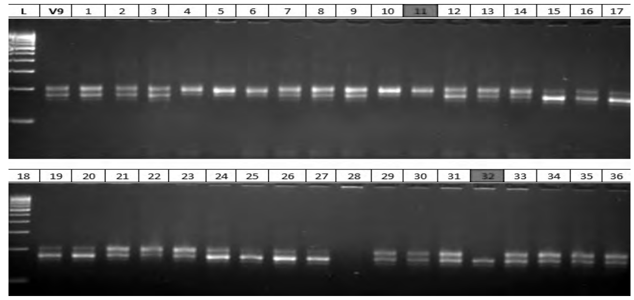

Figure 10. Molecular genetic purity test of V9: ICPL-87 with SSR Marker CcM0252

Legend: Lanes 11 and 32 are off-types; Lane 28 is a lapse

SSR marker CcM0252 and remaining 33 are homozygous with variety specific allele with molecular genetic purity (MGP) of 94.28 per cent (Figure 10).

In Bannur local, out of 15 seeds tested there was one lapse and two were off-types at lanes 10 and 11. Remaining 12 are homozygous with variety specific allele of the marker CcM0093 with MGP of 85.71 per cent (Figure 11). These observations were similar to the use of SSR markers in differentiating parental lines and hybrid [18] in case of paddy. Similar studies in pea also revealed that SSR markers are more precise than RAPD markers in determination of genetic diversity [25].

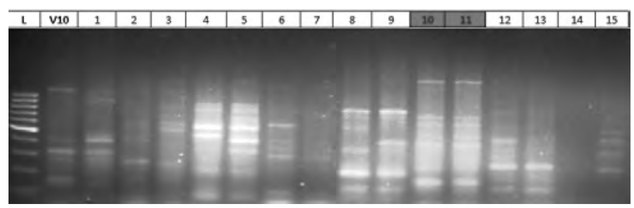

Figure 11. Molecular genetic purity test of V10: Bannur Local with SSR Marker CcM0093

Legend: Lanes 10 and 11 are off-types; Lane 14 is a lapse

Though RAPD markers were quite popular to identify genetic relations between genotypes till today AFLP markers were also considered as suitable tools for DNA fingerprinting in pigeon pea [26]. The successful use of SSR markers to test genetic purity in this study was similar to earlier studies in cotton [27] that used 22 SSR markers of different crop species origin to assess polymorphism through their SSR fingerprinting of 16 cultivated cotton genotypes. Also by [1] who succeeded in designing 113 pigeon pea SSR markers, 73 of which had amplified interpretable bands. 35 markers revealed polymorphism among 24 pigeon pea breeding lines. The SSR markers have many advantages over the other marker systems like high reproducibility, which would be the most important in genetic analysis. The SSR markers were co-dominant in nature and do not affected by environment or stage of the crop. Also it does not require template DNA to be ultra pure and small quantity can be used for molecular analysis [8].

While conducting molecular genetic purity test in pigeon pea in the present study using SSR markers and field grow out test some pre-determined number of random contaminants were added which help in validating results. This method of deliberate adding of contaminants is a practise reported by other researchers like Zhang *et al.* [27] accurately and effectively identifies the hybrid purity in a predetermined sample of cotton hybrids constituted by deliberately mixing seeds of parental lines.

Field Grow Out the Test for Validity of Molecular Genetic Purity Test Results

To validate the results of molecular genetic purity tests, a field grow out test was taken at UAS, Raichur demo farm. The field grow out test was taken for all the ten pigeon pea varieties adding the same contaminants as in the molecular genetic purity tests (Table 3). A total of 300-400 plants (including the random contaminants) per variety were evaluated as per the standard descriptors based on the morphological characters to calculate off type plants and genetic purity. The results of field grow out test were used to correlate with chemical and molecular genetic purity tests.

Correlation of Field Grow Out Test and Molecular Genetic Purity Test using SSR Markers

For the purpose of testing reproducibility and relating results of field grow out test and molecular genetic test using SSR markers, the data were subjected to paired ‘t’ test and correlation analysis. The details of this statistical analysis are furnished in table 4. It was observed that in the paired ‘t’ test the calculated t-value (1.66) is less than the table ‘t’-value (2.26) and there is no significant difference in the results obtained in molecular genetic purity test using SSR markers and genetic purity in field grow out test. Hence, both the methods can be used to assess the genetic purity of pigeon pea varieties. It was also observed that there is significant and positive correlation exist between these two methods (r= 0.77). Thus, genetic purity results obtained in molecular and field grow out tests was reliable and comparable to each other without any deviations.

The usefulness of DNA fingerprinting technique was first demonstrated in rice [22]. The seed produced need to be tested in field grow-out-test (GoT) for genetic purity before it is released for commercial cultivation thus confirming its genuineness and true-to-type characters. This may also add expenses of storage and handling to the final value of the seed. The DNA based approaches generally detect more polymorphism over other methods and constitute a new generation of genetic markers [26]. Conducting field GoT in pigeon pea is limited due to time constraints

Table 3. Field grow-out test of pigeon pea varieties

Sl. No	Variety	Seed Sample Size	Contaminants added *	Total Plants obtained	Total off types recorded	Off types %	Genetic Purity %
1	Maruti	400	55	368	31	8.42	91.58
2	TS3R	400	42	395	48	12.15	87.85
3	GRG-811	400	48	392	56	14.28	85.72
4	Asha	400	32	384	28	7.29	92.71
5	BSMR-736	400	35	379	42	11.08	88.92
6	GRG-833	400	52	391	41	10.48	89.52
7	GC-11-39	400	53	381	38	9.97	90.03
8	WRP-1	400	32	387	27	6.98	93.02
9	ICPL-87	400	33	394	36	9.14	90.86
10	Bannur Local	400	53	374	46	12.30	87.70

* Based on the off types detected in molecular genetic purity test using SSR markers

and dependent on environment [18] while, identification of simple sequence repeats (SSR) markers to assess genetic purity can speed up the seed testing and results can be available within shortperiod [21].

Table 4. Paired 't' test and correlation of molecular genetic purity and field grow out tests

Sl No	Variety	Genetic Purity in Molecular test	Genetic Purity in Field Grow Out test
1	Maruti	96.96	91.58
2	TS3R	88.23	87.85
3	GRG-811	87.50	85.72
4	Asha	95.83	92.71
5	BSMR-736	91.17	88.92
6	GRG-833	86.95	89.52
7	GC-11-39	92.85	90.03
8	WRP-1	92.00	93.02
9	ICPL-87	94.28	90.86
10	Bannur Local	85.71	87.70
	Mean	91.15	89.79
	Variance	15.35	5.43
t-test	df	9	
	Calculated t-Value	1.66	
	Table t-value	2.26	
	n=10	Hypothesised Mean Difference (α)=0.05	
Correlation analysis		r = 0.77	

The DNA methods have become an increasingly interesting future technology for varietal identification, purity testing and assessment of genetic diversity among the cultivars and fingerprinting of varieties. The commonly adopted approaches in the use of molecular markers could be probably based on screening of restriction fragment length polymorphism (RFLPs), polymerase chain reaction (PCR) based like random amplified polymorphic DNAs (RAPDs) or a combination of both like amplified fragment length polymorphisms (AFLPs) or it could be simple sequence repeat polymorphism based (SSRs) [28].

Cluster Analysis using Morphological Characters

The ten varieties were grouped in two clusters based on Euclidian^2 values in which varieties belonging to same cluster had smaller Euclidian^2 values than those belonging to other cluster. The distribution of varieties in to clusters is shown in figure 12.

Among the ten varieties, Maruti and Asha were related which are again related to GRG-811. The varieties ICPL-87 and Bannur Local were related which are again related to GC-11-39. All these six varieties (Maruti, Asha, GRG-811, ICPL-87, Bannur local and GC-11-39) are related and grouped as Cluster 1. The varieties TS3-R, GRG-833, WRP-1 and BSMR-736 were related and grouped as Cluster 2. All the varieties were analysed using Ward's minimum variance method based on the morphological characters.

The inference of these data indicates that, if more varieties are related then it is difficult to do a field grow out test to distinguish varieties based on morphological characters. If contaminants similar to varieties or other varieties appearing in variety as contaminant are tested for genetic purity, then also it is difficult to declare genetic purity results since morphological characters of a variety tested are similar to contaminants. Then to declare genetic purity based on molecular methods are the best option since it is more precise because of detection of contaminants at DNA level.

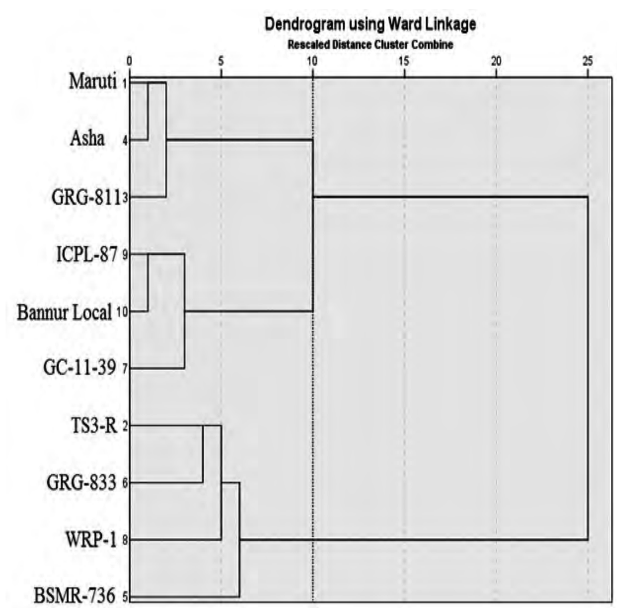

Figure 12. Dendrogram using scoring as per the DUS / morphological characters

Cluster Analysis using SSR Markers

The ten varieties were grouped in three clusters based on fixed Dice similarity coefficient of 3.71 in which varieties belonging to same cluster had smaller coefficient values than other clusters. The distribution of varieties in to clusters is shown in figure 13.

Among the ten varieties, in cluster 1, GRG-811 and Asha were related which are again related to Maruti which were related to TS3R. In cluster 2, GRG-833 and GC-11-39 were related and have similar similarity coefficient values which were related to BSMR-736 which were again related to WRP-1. In cluster 3 ICPL-87 and Bannur local varieties were related.

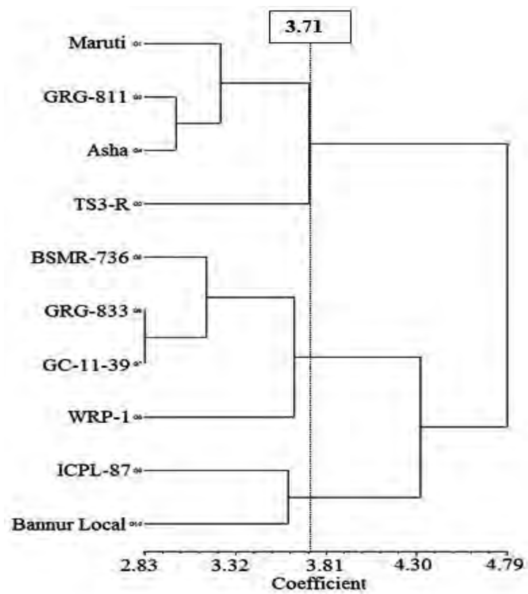

Figure 13. Dendrogram using binary scoring based on the SSR markers

CONCLUSION

Based on the results of present study, it is clear that genetic purity testing using SSR markers is more efficient and faster compared to field grow out test and chemical methods. These markers differentiate pigeon pea varieties with greater precision compared to field grow out test based on morphological characters. Hence, molecular genetic purity test using SSR marker can be used for declaring genetic purity results in short time and useful for the crops which are long durational like pigeon pea. This can be adopted for routine seed testing in place of field grow out test especially for crops like pigeon pea (often cross pollinated) subjected to change in laws of seed testing.

Despite of this, there are certain issues like standardization of seed sample size for molecular methods, sampling grow-out matrix for validity of molecular results and guidelines for use of SSR markers in genetic purity testing can be future line of work.

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