International Journal of Applied and Pure Science and Agriculture
ISSN Online - 2394-5532 Impact Factor: 3.762 by SJIF ISSN Print - 2394-823X

GENETIC DIVERSITY STUDIES IN PIGEON PEA OF HYBRIDS AND THEIR PARENTS

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Abstract

Genetic diversity studies of eleven hybrids and their twelve parents were also carried out using morphological and molecular markers. Ten morphological traits were used for morphological diversity studies. Out of ten, six traits showed polymorphism. Ten SSR primers of CCB series were used in molecular diversity analysis; all primers were found to be polymorphic among the parents and the hybrids. Similarity index values ranged from 0.166 to 1.00, 0.390 to 0.894, 0.375 to 0.875 for morphologically among parents, molecular level among parents and hybrids respectively. Dendrogram analysis showed five, seven and six clusters, at morphologically among parents, molecular level among parents and hybrids respectively. Present study will be help full for identification of genetically diverse parents and hybrids for broadening the genetic base and improving the breeding efficiency.

I. INTRODUCTION

DNA markers are unlimited in number and not influenced by environmental factors or the developmental stage of the plant, molecular markers have been widely used in genetic diversity analysis, establishment of relationship between cultivated species and their wide parents (Winter *et al.*, 1995).The desirable genetic variation in the cultivated pigeonpea is difficult to dissect due to narrow genetic base of pigeonpea germplasm. Plant evolution under domestication has led to increase productivity but at the same time it narrowed the genetic base of crop species. In an attempt to overcome these problems pigeonpea variants included in these studies are diverse in morphology than the improved cultivar used today. Though these variants are diverse in morphology; would these diversities be present at DNA level in variants and their F'_1s?.

II. MATERIALS AND METHODS

Twelve parents pigeonpea that including AKT-8811 and eleven pigeonpea variants i.e five height variants (Dwarf 30 cm, Dwarf 45 cm white seeded, Dwarf 45 cm brown seeded, Dwarf 60 cm and Dwarf 90 cm) and six leaf variants (Obcordifoliate, Oval shape, Unifoliate, Small, Sesamum and Gigas leaf variants) and their F'_1s were used to study the polymorphism at molecular and morphological level. Ten SSR primers commercially synthesized from OPERON technology USA were used for the present study is shown in table 1. DNA was extracted using Cetyltrimethyl ammonium bromide (CTAB) protocol given by Sharma *et al.* (2002) with some modifications. RNA was removed by giving Ribonuclease A treatment. RNAse A (10 mg/ml) was added to the DNA sample @ 100 µg/ml and incubated at 37^0C for 1 hour. DNA bands which were formed in the range of amplified product size given for particular primer (approximately 100 to 400 bp) were scored. These computations were performed using the statistical analysis package NTSYS-Pc (Numerical taxonomy System, version 2.02, Rohlf, 1990).

Presence of indeterminate growth habit, tall plant height, green stem, lanceolate leaf shape, trifoliate leaflet, yellow flower colour (dorsal side of standard), green with brown streaks pod colour, slight pod constriction, brown seed coat colour and oval

Table: 1 List of SSR primers used with their sequences

Sr. No.	Primer code	Primer sequence	Annealing Temperature (°C)
1)	CCB-1	L :AAGGGTTGTATCTCCGCGTG	57
		R :GCAAAGCAGCAATCATTTCG	
2)	CCB-2	L :CCATAATCCAATCCAAATCC	57
		R :AGAAGGCTTTCATGTAACGC	
3)	CCB-3	L :TCACAAAAACAAGTTGCCAC	52
		R :ATGACATGATTACGCCAAG	
4)	CCB-4	L :GGAGCTATGTTGGAGGATGA	57
		R :CCTTTTTGCATGGGTTGTAT	
5)	CCB-5	L :GACAATTTTGCATGCATTGC	57
		R :TTGCAAAACACTTGGTTGG	
6)	CCB-6	L :ACAATGCTAGGGAACACCGC	57
		R :TACCTTAACCCACAATGGCC	
7)	CCB-7	L :CAACATTTGGACTAAAACTG	55
		R :AGGTACCAATATCCAACTTG	
8)	CCB-8	L :TGCGTTTGTAAGCATTCTTCA	52
		R :ACTTGAGGCTGAATGGATTG	
9)	CCB-9	L :CACTTGGTTGGCTCAAGAAC	55
		R :GCCAATGAACTCACATCCTTC	
10)	CCB-10	L :CCTTCTTAACGTGAAATGCAAGC	50
		R :CATAACAATAAAAGACCTTGAATG	

shape seed scored as "1", and absence as "0" procedure for analysis is same as mention above for molecular diversity study.

III. RESULTS AND DISCUSSION

Polymorphism among parents on the basis of morphological markers

In present morphological markers studies, ten morphological markers viz., growth habit, plant height, stem colour, leaf shape, number of leaflet, flower colour (dorsal side of standard), pod colour, pod constriction, seed coat colour and seed shape are used for estimating genetic diversity among twelve parents including one female (AKT-8811) and eleven male parents. The similarity coefficient values ranged from 0.166 to 1.000. The relationship showed lowest similarity coefficient of 0.166 between Oval shape leaf variant with Dwarf variants 30 cm, 60 cm and 45 cm brown seeded and also between the Dwarf 45 cm white seeded and Small leaf variant. These similarity coefficient values indicated that maximum genetic diversity between height variants and leaf variants. The maximum similarity (1.000) was found between parent Dwarf 30 cm, Dwarf 60 cm and Dwarf 45 brown seeded; similarly the maximum similarity (1.000) was display by Obcodifoliate leaf variant with Sesamum and Gigas leaf variant; Sesamum leaf variant with Gigas leaf variant. In present study, ten morphological traits were used for diversity analysis, out of which six characters (growth habit, plant height, leaf shape, number of leaflet, pod colour and seed coat colour) were found diverse from each other and showed sixty percent variation among parental genotypes.

To determine the relationship between the parents, similarity matrix data were subjected to UPGMA cluster analysis to construct dendrogram. The dendrogram analysis exhibited total five clusters at 84 percent cut level of similarity. Clusters I and III contained only one parent i.e Oval leaf shape and Unifoliate leaf variant respectively. Unifoliate leaf variant had unique trait i.e. unifoliate leaflet which is not found in other parents and hence it was separated from other parents. Oval leaf variant had maximum number of contrasting traits (leaf shape, pod colour and seed coat colour) compared with to other parents. The cluster II contained four parents i.e only leaf variants such as Small, Gigas, Sesamum, and Obcordifoliate leaf variants. Cluster IV comprised two parents that included cultivar AK-8811 and Dwarf 90 cm which were similar in all qualitative traits except in plant height. Cluster V contained four parents which were height variants (Dwarf 30 cm, 45 cm brown seeded, 60 cm and 45 cm white seeded) except Dwarf 90 (Table 2). Similar results were

reported by Raghu *et al.* (2007) who evaluated morphological, SSR allele diversity and association mapping using 29 morphological traits and 15 SSR primers in 58 cassava accessions. In SSR analysis, a total of 71 alleles were generated and out of which 63 alleles were found to be polymorphic and the level of polymorphism was 86.56 per cent. Morphological descriptors revealed 6 clusters of accessions and while the SSR markers revealed 9 distinct clusters of accessions. The SSR allele indicated the existence of a wide genetic diversity among the cassava accessions compared to morphological data

Table: 2 Clustering pattern of parents on the basis of morphological markers

Details	Cluster	No. of Parents	Parents
	I	1	Oval shape leaf variants
	II	4	Small, Gigas, Sesamum and Obcordifoliate leaf variants
	III	1	Unifoliate leaf variant
	IV	2	AKT-8811 and Dwarf 90 cm
	V	4	Dwarf 30 cm, 45 cm brown seeded, 60 cm and 45 cm white seeded
Total	5	12	----------

Polymorphism among parents on the basis of SSR markers

All ten SSR primer used were found to be polymorphic. The total number of bands generated per primer pair ranged from 4 to 9, with an average of 5.9 bands per primer. Four amplicons were found in primer CCB - 2, CCB-4 and CCB-10. Highest numbers of bands (nine) were obtained in primer CCB-3. The total number of polymorphic bands produced by single primer varied from 3 (CCB-2, CCB- 6, CCB-7, CCB-9 and CCB-10) to 5 (CCB-1, CCB-5 and CCB-8) with an average of 3.8 polymorphic bands per primer. Ten SSR primer pairs produced total of 59 bands across 23 genotypes, of which 38 were polymorphic. The extent of polymorphism ranged from 37.50 per cent (CCB-7 and CCB-9) to 100 per cent (CCB-4 and CCB-5 and CCB-8) with an average of 64.40 per cent. The size of each amplified band ranged between 100-400 bp (Table 3).

Table : 3. Percent polymorphism of different SSR primers

Sr. No.	Primers	Total no. of amplicons	Polymorphic bands (Parents)	Percent of polymorphism (Parents)	Polymorphic bands (Hybrids)	Percent of polymorphism (Hybrids)	Product size (bp) range
SSR Polymorphism							
1	CCB -1	7	5	71.42	3	42.85	150 - 210
2	CCB -2	4	3	75.00	2	50.00	100 - 200
3	CCB -3	9	4	44.44	4	44.44	125 - 260
4	CCB -4	4	4	100	4	100	200 - 280
5	CCB -5	5	5	100	5	100	185 - 310
6	CCB -6	5	3	60.00	3	60.00	130 - 210
7	CCB -7	8	3	37.50	2	25.00	110 - 210
8	CCB -8	5	5	100	4	80.00	130 - 170
9	CCB -9	8	3	37.50	3	37.50	150 - 400
10	CCB -10	4	3	75.00	2	50.00	150 - 250
	Total	59	38	-	32	-	-
	Average	5.9	3.8	64.40	3.2	54.23	-

Singh *et al.* (2008) studied twenty two SSR markers for different crop species were used to assess polymorphism of 16 cultivated pigeonpea genotypes. Four hundred twenty five bands were amplified in all the sixteen genotypes. A total of 46 SSR fragments were amplified. Eight primers showed 100 per cent polymorphism. Datta et *al.* (2010) used 22 SSR markers to genetically differentiate two major pulse crops possessing different important traits and observed polymorphism

93 per cent. Total number of alleles produced per primer in present study was similar to result of Saxena et *al*. (2010) concluded that thirteen SSR markers were polymorphic amongst 32 cultivated and eight wild pigeonpea genotypes representing six *Cajanus* species.

These markers amplified a total of 72 alleles ranging from two to eight alleles with an average of 5.5 alleles per locus. Sheikh *et al.* (2011) analyzed 80 RAPD markers in ten different genotypes and observed a total of 702 bands, out of which 544 were polymorphic. This evinced on an average 7.55 polymorphic bands/primer; though the average number of amplified bands per primer were 8.77. The size of amplified bands ranged from 100 bp to 2850 bp. Kale *et al* (2008) studied 29 pigeonpea genotypes. Out of 12 SSR primers used for SSR analysis, 9 yielded good polymorphism. Total of 37 alleles were obtained with an average of 4.1 alleles per locus. Gel photographs obtained in the present study of SSR primers i.e. CCB-3 and CCB-5 are shown from Plate 17 and 18.

The binary data obtained through the SSR profile of 12 parents were subjected for obtaining similarity matrix. The similarity coefficient among parents in the present investigation ranged from 0.394 to 0.894. The lowest similarity coefficient of 0.394 was found between the parent Dwarf 45 cm white seeded (height variant) with two parents i.e. Gigas leaf variant and AKT-8811. A lowest similarity value between them was due to morphological diversity of height variants with leaf variants and also parent AKT-8811 cultivated variety. The maximum similarity of 0.894 was found between obcordifoliate leaf variant and oval shape leaf variant because both being the leaf variants. Gupta and Singh (2010) determined molecular diversity among twenty maize genotypes using ten simple sequence repeat (SSR) primer sets. The pair wise similarity was calculated by Jacquard's similarity coefficients, which ranged from 0.12 to 0.73 with an average similarity of 0.42.The minimum similarity (0.12) was found between two pairs, HUZM-356 with HUZM-47 and HUZM-53 with HUZM147 which are more diverse. The less diversity and maximum similarity (0.73) was found between HUZM-147 with HUZM-36 genotype.

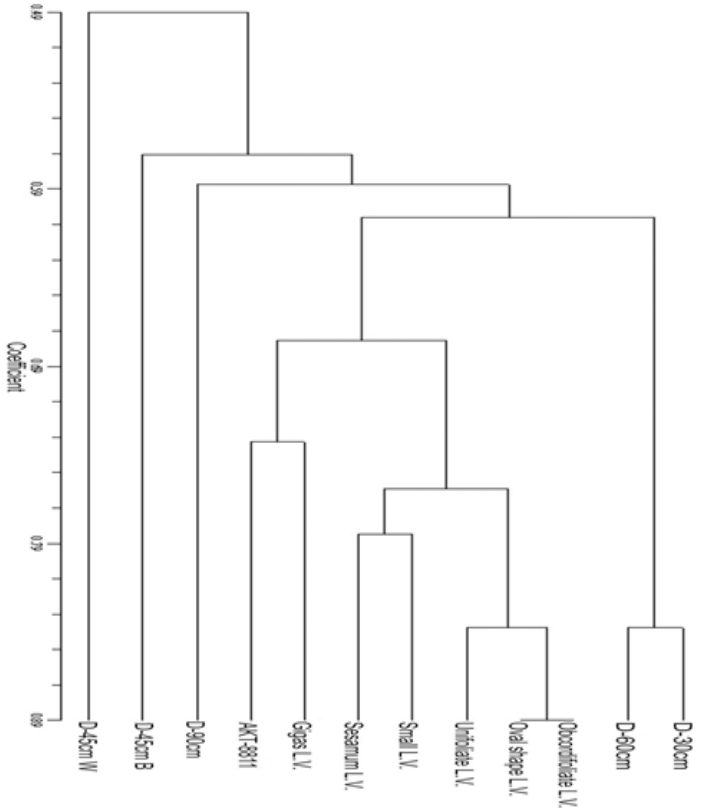

Fig. 1: Dendrogram of parents based on Jaccard's similarity coefficient using SSR markers

Dendrogram was constructed using UPGMA method of cluster analysis based on similarity coefficients generated by 10 SSR primers as shown in Fig.1. The dendrogram generated total seven clusters at 75 percent cut level of similarity. Clusters I, II, III, IV and V contained only one parent i.e. Dwarf 45 cm white seeded, Dwarf 45 cm brown seeded, Dwarf 90 cm, AKT-8811 and Gigas leaf variant respectively.The cluster VI contained all leaf variants except Gigas leaf variant. The cluster VII contained two height variants i.e. Dwarf 30 cm and Dwarf 60 cm (Table 4). From the results obtained through SSR analysis, it is clear that SSR data efficiently separated parents. Similar types of finding were reported by Rupakula *et al*. (2009) who observed that wild and cultivated *Cajanus* accessions belonging to different species were clustered into four distinct major groups largely based on the interspecific differences. *C. scarabaeoides* accessions derived from same geographical origins formed one group. Verma *et al* (2010) stated that the clustering pattern had six major groups of which non-nodulating; medium nodulating and high nodulating parents were grouped into three distinct clusters.

Table : 4 Clustering of parents on the basis of SSR markers

Details	Cluster	No. of Parents	Parents
	I	1	Dwarf 45 cm white seeded
	II	1	Dwarf 45 cm brown seeded
	III	1	Dwarf 90 cm
	IV	1	AKT-8811
	V	1	Gigas leaf variant
	VI	5	Sesamum, Small, Unifoliate, Oval shape and Obcordifoliate leaf variants
	VII	2	Dwarf 30 cm and Dwarf 60 cm
	Total	12	----------

Polymorphism among hybrids on the basis of SSR markers

A total of ten SSR primers were screened, all of them showed consistently good bond profile. In ten SSR primers, total 59 bands were amplified out of which 32 were polymorphic. Total number of bands and average bands per primers were 5.9 and 3.2 respectively. The size of bands ranged from 100 bp to 400 bp. Primer CCB-3 amplified highest alleles (9) as compared to other primers. The highest percent of polymorphism was recorded in two primers CCB-4 and CCB- 5 (100 per cent) and lowest of 25 per cent recorded in CCB-7. The total number of bands, number of polymorphic bands and percentage of polymorphism of different SSR primer pairs in pigeonpea genotypes is shown in Table 4**.** Similar results of SSR polymorphism among hybrid genotypes were reported by Cholastova *et al.* (2011). Out of 43 SSR primers, 12 polymorphic SSRs were selected. A total of 49 polymorphic bands were amplified in maize hybrids using these SSR markers. On an average, 4.08 alleles were obtained per locus. Sun *et al*. (2001) used 17 primers with DNA obtained from 37 hybrids. A total of 79 bands were scored and averages of 4.6 alleles were amplified per primer.

The binary data obtained through the SSR profile of 11 hybrids were subjected to analysis for calculating similarity matrix. The similarity matrix was calculated among the hybrids. The lowest similarity coefficient of 0.375 was found between Sesamum leaf variant F_1 (AKT-8811 × Sesamum leaf variant) and Dwarf 45 cm brown seeded F_1 (AKT-8811 × Dwarf 45 cm brown seeded). Their parent Sesamum leaf variant and Dwarf 45 cm brown seeded were also diverse showing similarity coefficient 0.50 and 0.421 at morphological and molecular level respectively. It revealed that parents diverse at morphological and molecular level, their F'_{1S} produced also showed diversity between them.The maximum similarity of 0.875 was found between Sesamum leaf variant F_1 (AKT-8811 × Sesamum leaf variant) and Gigas leaf variant F_1 (AKT-8811 × Gigas leaf variant). Their parents Sesamum leaf variant and Gigas leaf variant also showed maximum similarity coefficient 1.000 and 0.657 at morphological and molecular level respectively. It concluded that parents showing

maximum similarity at morphological and molecular level, their F'_{1S} produced also showed maximum similarity between them.

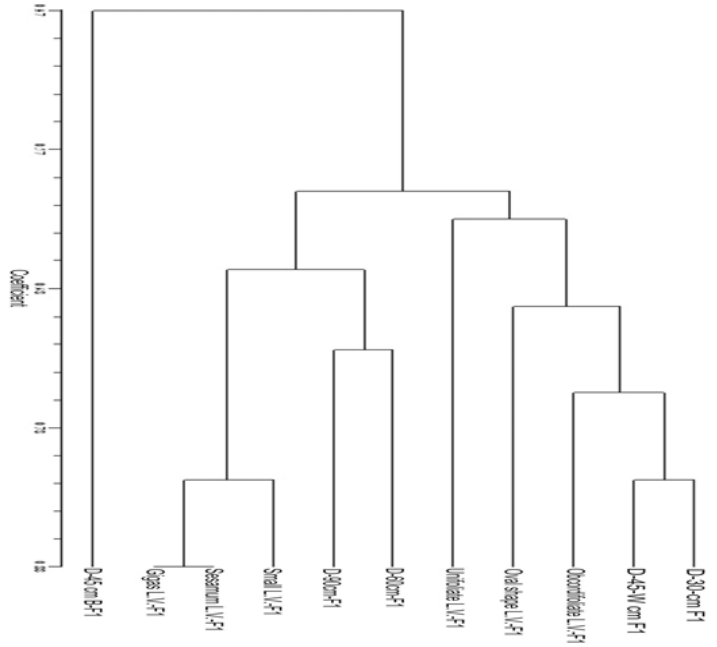

Fig. 2 Dendrogram of hybrids based on Jaccard's similarity coefficient using SSR markers

To determine the relationship between the hybrids similarity matrix data were subjected to UPGMA cluster analysis to construct dendrogram as shown in Fig. 3.

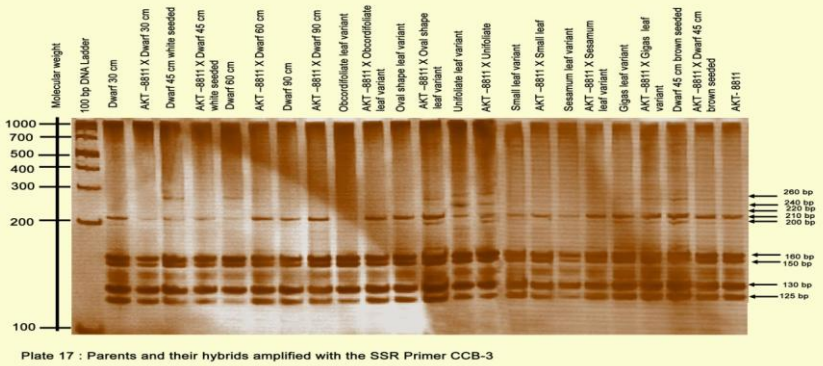

Plate 17 : Parents and their hybrids amplified with the SSR Primer CCB-3

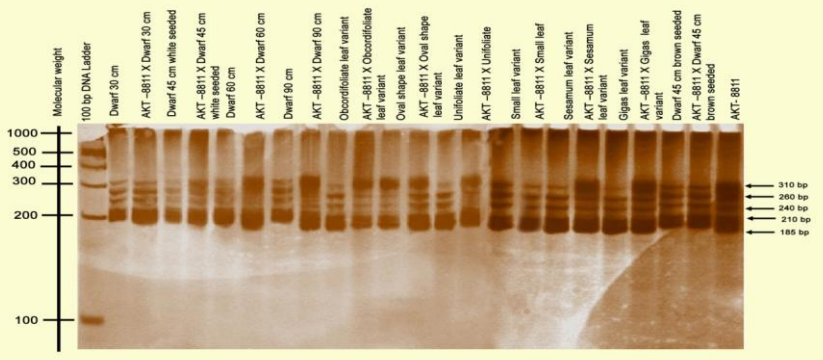

Plate 18: Parents and their hybrids amplified with the SSR Primer CCB-5

The dedrogram analysis showed six clusters at 71 percent cut level of similarity. Clusters I, IV, and V included Dwarf 45 cm brown seeded F_1 (AKT-8811 × Dwarf 45 cm brown seeded), Unifoliate leaf variant F_1 (AKT-8811 × Unifoliate leaf variant) and Oval shape leaf variant F_1 (AKT-8811 × Oval shape leaf variant) respectively. Their parent Dwarf 45 cm brown seeded also present separate in cluster II in dendrogram generated by molecular studies among parents (Fig. 2). Parents Unifoliate leaf variant and Oval shape leaf variant also found separated as single parents in cluster III and I respectively, as shown in dendrogram generated by morphological data (Fig. 1).The cluster II contained three hybrids Gigas leaf variant F_1 (AKT-8811 × Gigas leaf variant), Sesamum leaf variant F_1 (AKT-8811 × Sesamum leaf variant) and Small leaf variant F_1 (AKT-8811 × Small leaf variant). The cluster III comprised two hybrids Dwarf 60 cm F_1 (AKT-8811 × Dwarf 60 cm) and Dwarf 90 cm F_1 (AKT-8811 × Dwarf 90 cm). The cluster VI included three hybrids namely, Dwarf 30 cm F_1 (AKT-8811 × Dwarf 30 cm), Dwarf 45 cm white seeded F_1 (AKT-8811 × Dwarf 45 cm white seeded) and Obcordifoliate leaf variant F_1 (AKT-8811 × Obcordifoliate leaf variant). Details of dendrogram generated through SSR markers among hybrids are given in Table 5.

Table: 5 Clustering of hybrids on the basis of SSR markers

Details	Cluster	No. of Hybrids	Hybrids
	I	1	Dwarf 45 cm brown seeded F_1
	II	3	Gigas leaf variant F_1, Sesamum leaf variant F_1 and Small leaf variant F_1,
	III	2	Dwarf 90 cm F_1 and Dwarf 60 cm F_1
	IV	1	Unifoliate leaf variant F_1
	V	1	Oval shape leaf variant F_1
	VI	3	Obcordifoliate leaf variant F_1, Dwarf 45 cm white seeded F_1 and Dwarf 30 cm F_1
Total	6	11	----------

Similar results of clustering of hybrids were reported by Smith *et al.* (2010) Sixty-three sorghum hybrids that are, or have been, widely used in the United States were profiled using 167 simple sequence repeat (SSR) markers. Genetic distance and cluster analysis determined that 43 (68 per cent) of the hybrids formed seven groupings, with each group containing hybrids commercialized by a single breeding organization. Groupings were also reflective of known pedigrees. Hybrids released by different organizations were not very closely associated with one exception of two hybrids. Groupings had limited association with the decade that the hybrid was released. Numbers of alleles per locus have been constant during the past three decades with gain in new alleles being balanced by loss of other alleles. Hybrids released during the 2000s showed the least number of new alleles compared to the previous two decades.

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