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Genetic Diversity Assessment in Pigeonpea Cultivars Using Microsatellite (SSR) Markers

Nagaraj Hullur[1*], B.N. Radha[2], B. Basavaraja[1], B.C. Channakeshava[3] and M. Byregowda[4]

[1]Department of Seed Science and Technology, College of Agriculture, Karekere, Hassan-573225, India
[2]Department of Seed Science and Technology, College of Sericulture, Chintamani, India
[3]Department of Seed Science and Technology, College of Agriculture, UAS, GKVK, Bengaluru, India
[4]AICRP on Pigeonpea, UAS, GKVK, Bengaluru, India
**Corresponding author*

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A B S T R A C T

Genetic diversity among 20 cultivars of pigeonpea (*Cajanus cajan* (L) Millsp.) analysed using twelve SSR markers. Although most of the cultivars indicated polymorphism for the studied primers, cultivars BRG-2, BRG 5, GRG 818, HY3C, ICP 8863, ICPL 87119 and JKM 189 could amplify specific unique amplicon at higher molecular weight (138-148 bp) when primer CCB-8 was used. Similarly cultivars AKT 08-02, CoRG 9701, GRG 818, JKM 189, JKM 206, TS 3R, TTB, WRG 173 and WRP 1 could amplify specific unique amplicon at higher molecular weight (155-180 bp) when CCB-9 primer was used. In case of primer PB-3, cultivars AKT 08-02, BRG 1, BRG 3, CoRG 9701, GRG 822, ICP 8863 could amplify specific unique amplicon at higher molecular weight (280-340 bp) and primer PB-8 could amplify cultivars BDN 2, BRG 1, BRG-2, BRG 3, BRG 4, JKM 189, JKM 209, TS 3R, TTB-7 at higher molecular weight (305-320bp). Among the twelve primers (CCB-10, CCB-3, CCB-6, CCB-7, CCB-8, CCB-9, PB-1, PB-3, PB-7, PB-8, PB-9, PB-10) used in the present study for genetic diversity assessment CCB-8, CCB-9, PB-3 and PB-8 can be useful in detecting the genetic structure of the plants rapidly and easily by PCR technique via marker aided selection in early stages of growth.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Mills.] is one of the important grain legumes of India, which plays an important role in food security, balanced diet and alleviation of poverty (Rao *et al.,* 2002). It contains 22-24 percent protein, which is rich in essential amino acids and abundant in minerals, vitamins, iodine and iron (Saxena, 2002).

It is also used as fuel wood and for rearing of lac insects (Zenghong *et al.,* 2001). Besides, its usages as hedges and windbreaks and for green manuring and house roofing are also documented. It has an inherent ability to withstand environmental stresses especially drought due to its paraheliotrophic nature (Nene and Shaila 1990).

DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983). To quantify the genetic diversity among and similarity between and among the genotypes, simple sequence repeats (SSR) are the marker of choice for genetic studies like genetic diversity assessment, genetic mapping, population genetics and marker-assisted selection by virtue of their extreme polymorphism, ubiquitous presence and co-dominant inheritance (Rafalski and Tingey, 1993, Gupta *et al.,* 1996, Jarne and Lagoda, 1996) and robust, reproducible, hypervariable, informative and reasonably easy-to-use properties (Powell *et al.,* 1996). This study was undertaken to assess the genetic diversity among the pigeonpea cultivars and to identify the molecular markers for cultivars used.

Materials and Methods

The twenty pigeonpea genotypes *viz*., AKT 08-02, BDN-2, BRG-1, BRG-2, BRG-3, BRG-4, BRG-5, CoRG 9701, GRG-818, GRG-822, Hy 3C, ICP 8863, ICPL 87119, JKM-189, JKM-206, JKM-209, TS 3R, TTB-7, WRP-1 and WRG-173 used for the present investigation were obtained AICRP on pigeonpea, UAS, GKVK, Bengaluru, India. The seeds were surface sterilized with 1 % (w/v) mercuric chloride and 70 % ethanol and then rinsed with de-ionized water and grown for two to three weeks in polythene bags.

The genomic DNA extraction was done using Cetyl trimethyl ammonium bromide (CTAB) method described by Cuc *et al.,* (2008) with few modifications as detailed below. 70-100 mg fresh leaf samples of 15 days old seedlings collected and were cut into pieces and ground into fine powder by using liquid nitrogen to a fine paste with the extraction buffer 450 µl for each sample [1.4 M NaCl; 20 mM EDTA; 2-3 % w/v CTAB and 0.2 % β – Mercaptoethanol (added at the time of use to homogenized)]. Another 450 µl of extraction buffer was added again and mixed well. From this around 400 µl homogenized solution was taken into an eppendorf tube, incubated at 65 °C for 10 min with shaking at periodical intervals. 450 µl of chloroform ($CHCl_3$) and iso-amyl alcohol in a 24:1 ratio was added and mixed thoroughly by inverting twice to emulsify both the components followed by centrifugation at 5500 rpm for 10 min at room temperature. The aqueous layer (300 µl) was transferred to fresh strip tubes.

The supernatants were treated with 210 µl of chilled iso-propyl alcohol and incubated at -20 °C for 30 minute. After centrifugation at 8000 rpm for 15 min, the supernatant was removed and the DNA pellet was washed twice with 70 % ethanol and dissolved in TE buffer (10 mM tris and 1 mM EDTA; pH 8.0) after drying for 15 min. The extracted DNAs were quantified by agarose gel (0.8 %) electrophoresis using uncut lambda DNA as standard.

The SSR reaction mixture consisted of 1.0 µl of template DNA, 0.3 µl forward primer, 0.3 µl reverse primer, 2.0 µl dNTPs, 2.0 µl Taq, 2.0 µl of 1X PCR buffer (10 M Tris pH 8.0, 50 mM KCl, 1.8 mM MgCl2 and 0.01 mg / ml gelatin) and 14.1 µl of sterile water in a volume of 20 µl. To this one drop of mineral oil (Sigma) was added on to the reaction mixture. The SSR primers used in the study are given in Table 1.

Amplification conditions were maintained at 94 °C for 5 min, and 45 cycles at 94 °C for 1 min (denaturation), varied temperature as per particular primer (Table 1) for 1 min (annealing), 72 °C for 1 min (elongation) followed by final extension at 72 °C for 10 min.

The PCR products were resolved on 10 % PAGE followed by silver staining as per the

procedure given by Tagelstrom (1992). After the run, the gel was viewed under UV light and the DNA banding pattern was recorded directly and photographed using Polaroid Camera in gel documentation system.

Statistical analysis

The data analysis was performed using the SPSS (Statistical Package for the Social Sciences). It is a common estimator of genetic relationship. Genetic relationships among the cultivars were obtained using similarity matrices by constructing dendrogrammes through Ward's method.

Results and Discussion

Among the twelve SSR primers (CCB-10, CCB-3, CCB-6, CCB-7, CCB-8, CCB-9, PB-1, PB-3, PB-7, PB-8, PB-9, PB-10) utilized in the study, four CCB-9, PB-8, PB-3 and CCB-8 generated polymorphic banding pattern (Fig. 1). Although most of the cultivars indicated polymorphism for the studied primers, cultivars BRG-2, BRG 5, GRG 818, HY3C, ICP 8863, ICPL 87119 and JKM 189 could amplify specific unique amplicon at higher molecular weight (138-148 bp) when primer CCB-8 was used. Similarly cultivars AKT 08-02, CoRG 9701, GRG 818, JKM 189, JKM 206, TS 3R, TTB, WRG 173 and WRP 1 could amplify specific unique amplicon at higher molecular weight (155-180 bp) when CCB-9 primer was used.

In case of primer PB-3, cultivars AKT 08-02, BRG 1, BRG 3, CoRG 9701, GRG 822, ICP 8863 could amplify specific unique amplicon at higher molecular weight (280-340 bp) and primer PB-8 could amplify cultivars BDN 2, BRG 1, BRG-2, BRG 3, BRG 4, JKM 189, JKM 209, TS 3R, TTB-7 at higher molecular weight (305-320bp).

A high level of polymorphism at the SSR loci has been reported in pigeonpea (Ratna *et al.,* 2014; Adna *et al.,* 2011).

The primers identified in the present study *viz*., CCB-8, CCB-9, PB-3 and PB-8 can be useful in detecting the genetic structure of the plants rapidly and easily by PCR technique via marker aided selection in early stages of growth (Akagi, 1995) otherwise it is very tedious, time, land, and labour intensive especially in the long duration crop like pigeonpea.

Similarity index is the indicative of genetic relatedness which gives idea about the genetic diversity present in pigeonpea cultivars. The genetic relationship between twenty pigeonpea genotypes was assessed on the basis of Ward's similarity coefficients using SPSS programme. Based on dendrogram cultivars were grouped in to two major clusters (Fig. 2).

The first major cluster consisted of 14 cultivars and remaining cultivars were grouped in to second major cluster. Most of the studied cultivars were shown distinctiveness for SSR markers. Cluster analysis based on microsatellite allelic diversity, clearly demarcated the pigeonpea cultivars into different groups.

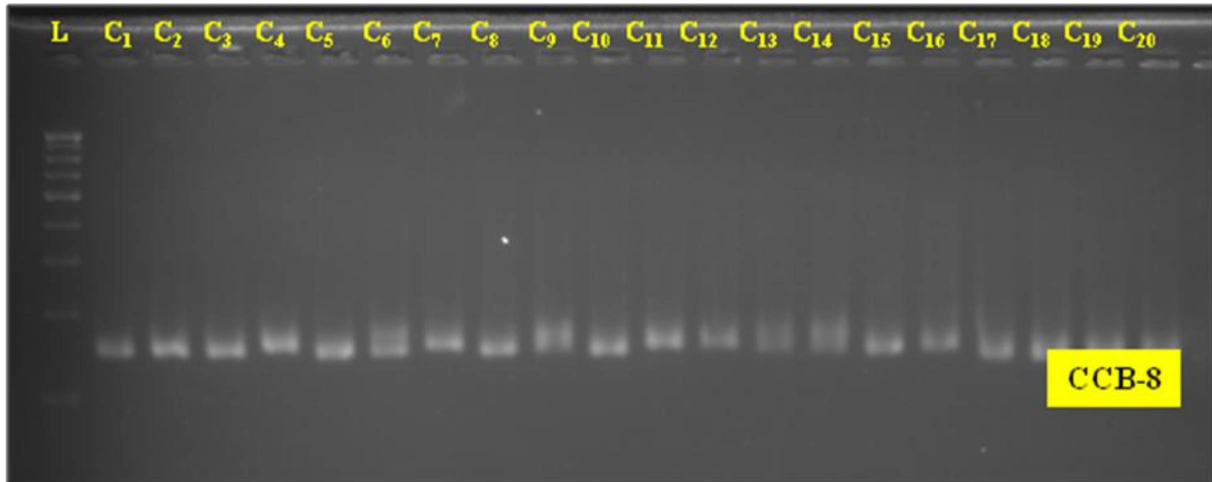
Reason for detected diversity might be due to the problem of linkage drag during the development of genotypes. This undetectable variability might have been magnified by molecular markers which were not expressed at phenotypic level.

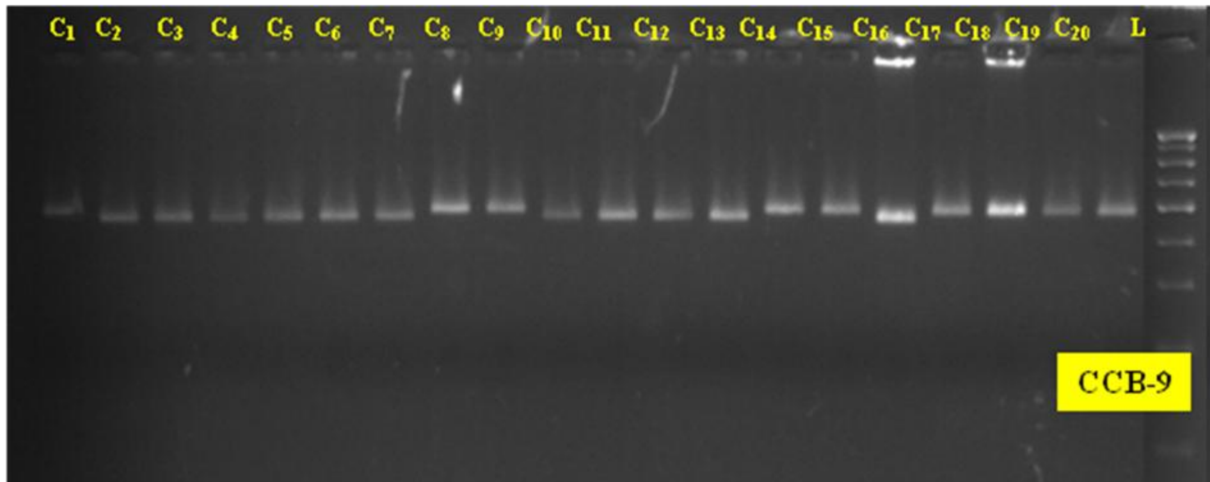
Another possible reason for the detected variability at molecular level might be due to conserved sequence of the isogenic lines which may not express at morphological level. Brown-Guedira *et al.,* (2000) also recorded similar results in soybean.

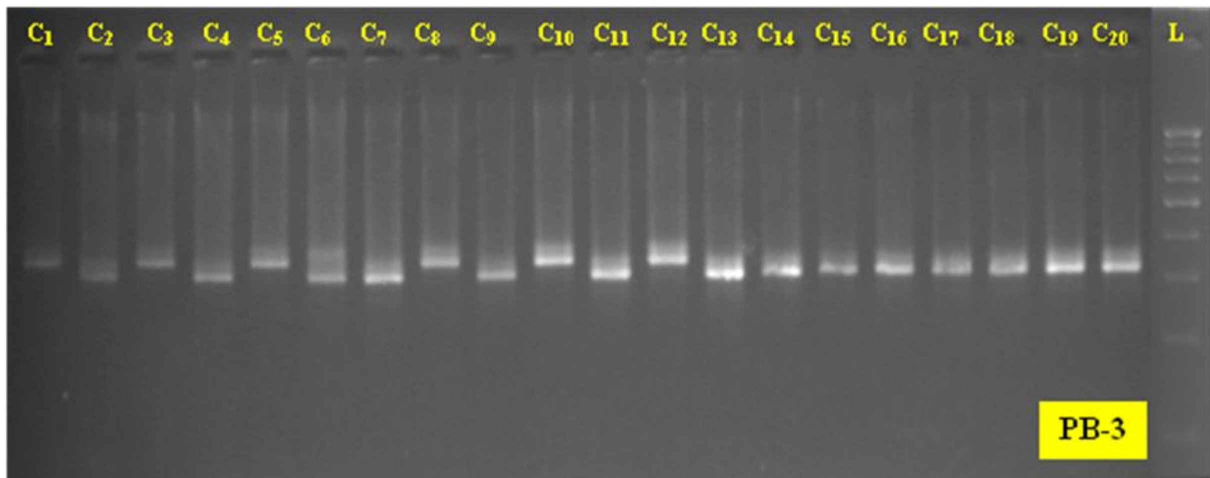
Table.1 List of SSR primers used with their sequences, annealing temperature and amplification range

Sl. No.	Primer code	Primer sequence		Annealing Temp (°C)	Amplification range (bp)
		Forward	Reverse		
1	CCB-10	CCTTCTTAACGTGAAATGCAAGC	CATAACAATAAAAGACCTTGAATGC	50	244-250
2	CCB-3	TCACAAAAACAAGTTGCCAC	ATGACATGATTACGCCAAG	52	150-156
3	CCB-6	ACAATGCTAGGGAACACCGC	TACCTTAACCCACAATGGCC	57	202-208
4	CCB-7	CAACATTTGGACTAAAACTG	AGGTACCAATATCCAACTTG	55	150-158
5	CCB-8	TGCGTTTGTAAGCATTCTTCA,	ACTTGAGGCTGAATGGATTG	52	138-148
6	CCB-9	CACTTGGTTGGCTCAAGAAC	GCCAATGAACTCACATCCTTC	55	155-180
7	PB-1	CGGGCTTCCTTTTCTTCTCT	AAAACCCCGAAAACACCATT	55	220-235
8	PB-3	CTTCCCCCAACTAAGATCCA	GTTCGTTCTCTTTAATTGACTTGC	55	280-3440
9	PB-7	GTCGGGGCGTGTAAGTCATA	CCGAAATAAGGATGGCAAAT	48	170-230
10	PB-8	GTCTTTGAGGGACGGAACC	GGGGCGGGGAAAGTACATA	55	305-320
11	PB-9	CCAAGAAAAGGTGCTCCAAGT	TTGCTTCTTTTCTCGCTTGC	53	180-190
12	PB-10	TGATAGGACCAACGACA	AGCGTTGACTCCTCCCTCTT	53	155-185

Fig.1 SSR banding profile of pigeonpea cultivars AKT 08-02, BDN-2, BRG-1, BRG-2, BRG-3, BRG-4, BRG-5, CoRG 9701, GRG-818, GRG-822, Hy 3C, ICP 8863, ICPL 87119, JKM-189, JKM-206, JKM-209, TS 3R, TTB-7, WRP-1 and WRG-173 with primers CC-8, CCB-9, PB-3 and PB-8







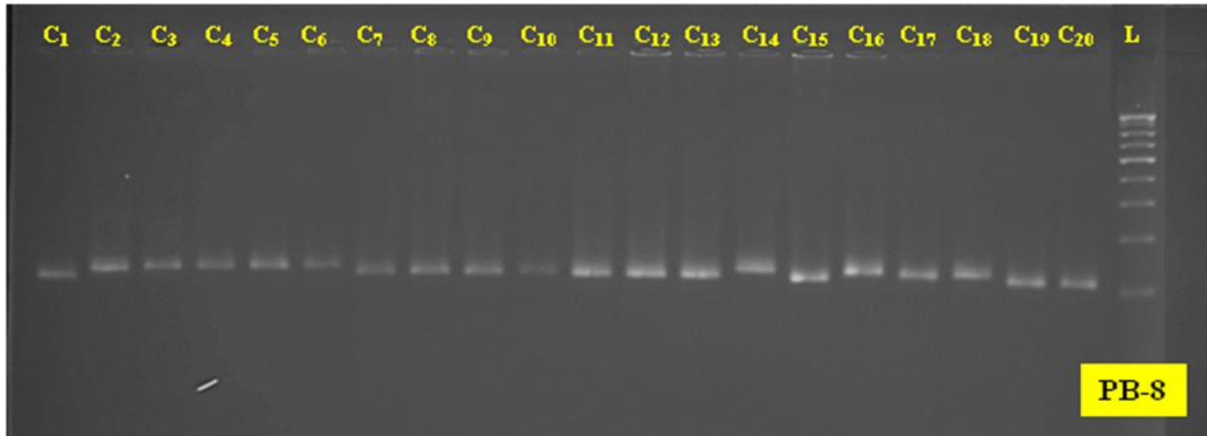
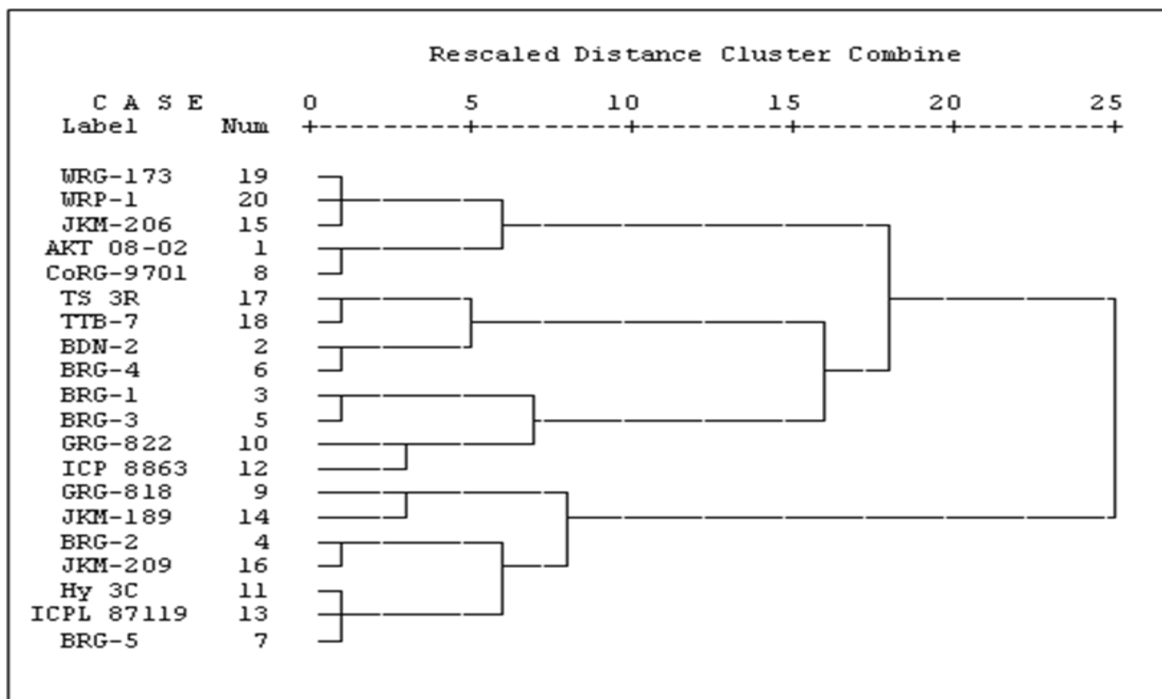


Fig.2 Dendrograms constructed using Ward's method and clustering for 20 pigeonpea genotypes based on SSR markers



The results would be very helpful for identification of cultivars there by maintaining the genetic purity of cultivars. Further, the primers identified in the present investigation would be useful in the identification and grouping of pigeonpea cultivars at DNA level during early growth stages, which in turn, would be an alternative to grow out test of these cultivars in commercial seed production programme.

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