(Article)

HYBRID TESTING IN PIGEONPEA USING DNA FINGERPRINTING BY SSR-MARKERS

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Abstract: Pigeonpea (*Cajanus cajan* (L.) of Fabaceae family belongs to genus *Cajanus* usually grown in semi-arid tropics of Asia and Oceania, Africa and America. This crop has been a best source for improving food and soil quality amongst farmers. However, its seed have been always questioned for purity. This problem is managed by using polymorphic SSR markers. In present study, a DNA fingerprints generated by seven SSR markers and hybrid testing is performed on Pigeonpea test samples along with parental lines. The seed samples of pigeonpea were germinated in laboratory and three week old leaves samples were used for DNA isolation by CTAB method. A total of 9 alleles were observed in three test samples using three primers out of seven primers. The screening of the allelic data associated with the three cultivated varieties, revealed markers (CcM0246) displayed unique allelic profiles for one variety. Yet, the genetic fingerprinting data is not well resolved to potentially distinguished two bands of hybrid that are merely of 4–8 bp to confirm hybrid testing of seed. Hybrid testing of may be confirmed including more SSR primers prepared from genomic DNA of pigeonpea.

Keywords: DNA Fingerprinting, SSR-markers, Gel electrophoresis, Hybrid testing and Pigeonpea.

1. Introduction

Pigeonpea, *Cajanus cajan* [L] is a drought tolerant crop and one of the most important legumes grown in the tropics and sub tropics, popularly known as red gram (*arhar dal / tur dal*). It is largely used as supplement cereal because it is a rich source of protein for humans. Pigeonpea is a major food legume in South Asia and East Africa. However, India is the world's largest producer (3.3 mha). Globally the cultivation of pigeonpea is about 4.92 mha which got sixth rank following other legume plants [1]. The protein percentage in seeds is about 20-22% in this crop with enough amount of essential amino acids. Pigeonpea fixes atmospheric nitrogen and improves the quality and structure of soils because of deep root system while the perennial type of pigeonpea provide fuel wood, food and fodder. But the crop suffers from several biotic and abiotic stress, production suffers greatly from mixing of low quality and contaminated seeds. So, there is need to include new genetic resources in pigeonpea breeding with the help of modern tools of Biotechnology to overcome the yield constraints. Marker assisted breeding is one of the solution to achieve higher yield. This plays vital role in assessing seed purity {1,2].

Microsatellites or simple sequence repeats (SSRs) are short tandem repeats of 1-6 neucleotides evenly separated in genome and are present in all eukaryotes. This are standard DNA markers for evolutionary and genetic studies. SSR markers has multi-allelic nature, co-dominant transmission, relative abundance, extensive genome coverage, high reproducibility, simple detection and only a small amount of template DNA require to the extraordinary increase of interest in SSRs in many

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organisms [1,2]. As compare to other cereals and legumes, the development and use of molecular markers in pigeonpea is limited. Thus, in present study, we used SSR markers on pigeonpea varieties to provide evidence of their purity.

2. Materials and Methods

Plant material

The test pigeonpea samples, parental lines and control hybrid were obtained from Seed Testing Laboratory, Nagpur. The cultivar name is ICPH 2740 (Hybrid) along with parental lines (ICPA2047 and ICPR 2740) [A-line (female) and R-line (male)] and three sample set: sample 1: Lot no. AP-DSR-1609-16H, sample 2: Lot no. 01-P39-08-03-07 and sample 3: Lot no. 01-P39-08-03-11. Individual seeds from each sample as well as reference hybrid and parental lines were kept for germination in seed germination paper.

Reagent and chemicals

The reagents and chemical used for preparation of CTAB buffer (100 ml) are as fallows CTAB (Cetyl- trimethyl- ammonium bromide) 2.0 gm, 0.5 M EDTA (pH 8.0) 4 ml, 1 M Tris Cl (pH 8.0) 10 ml, 5.0 M NaCl 28 ml and H_2O 40 ml, PVP 40 (polyvinyl pyrrolidine) 1 gm, adjust all to pH 5 with HCL and make volume up to 100 ml by double distilled water, NaOH 10 M (pH adjustment of buffer), pure cold (-20°C) isopropanol, chloroform: isoamyl alcohol (24:1 v/v), 70% ethanol, Enzyme: Dream Taq DNA polymerase (Himedia), Buffer: 10x Dream Taq buffer (Himedia), Nucleotides: dNTPs (G, A, T, C) 2 mM and SSR-marker primers, TAE buffer, agarose gel and ethidium bromide [3]. The test sample 1: Lot no. AP-DSR-1609-16H, sample 2: Lot no. 01-P39-08-03-07 and sample 3: Lot no. 01-P39-08-03-11.

SSR- marker Primers

The SSR primers used in the present study were obtained from ICRISAT. The details of SSR-markers such as sequences, melting temperatures and expected amplified DNA size in hybrid and parental lines are represented in table 1.

Table 1. The SSR markers, sequences (F/R), their length, melting temperatures and expected amplified DNA size for hybrid/ male (R)/ female (A)/test samples of pigeonpea are shown.

Sr no	Primer name	Sequences (5 3)	Length (bp)	Tm	Expected amplified bands
1	CcM0246	ATGGAGCCAAAGTGTCCAAG (F)	20	52°C	251bp (A)
		ATTGATGGTGTTGTGGCAGA (R)	20	50°C	245bp (R)
2	CcM0516	ATTGATGGTGTTGTGGCAGA(F)	20	50°C	207 bp (A)
		TTCGTGACACTCACTGGTCC (R)	20	54°C	211 bp (R)
3	CcM0008	CGGTGAAAAGGGTCAATGAG (F)	20	52°C	208 bp (A)
		CAAAATTAAAGCCTACTTGTTTTACGA (R)	27	51°C	202 bp (R)
4	CcM0207	TTTTGGCGGTCATTTTAACC (F)	20	48°C	256 bp (A)
		TAAGTCGGGAGCAACACTGA (R)	20	52°C	254 bp (R)
5	CcM0494	ACGTGAAAAATCCGCAACTT (F)	20	48°C	129 bp (A)
		GTCTGTGTTTCAAAATCCAACTT (R)	23	50°C	138 bp (R)
6	CcM0133	GTTGTCCCATTTTGACCTCC (F)	20	52°C	200 bp (A)
		CCATAATCCAATCCAAATCCA (R)	21	49°C	208 bp (R)
7	CcM0948	GCACAGGTCACGTCTGTACC (F)	20	56°C	235 bp (A)
		CATTTTCCCACCTTTCCTGA	20	50°C	239 bp (R)

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DNA extraction

The genomic DNA was extracted from three week old leaves of the individual seedling from the control hybrid, parental lines and test samples of pigeonpea to be tested, using CTAB (cetyl trimethylammonium bromide) DNA isolation method as follow,

1. Fresh 100-500 mg of young leaf samples of pigeonpea were taken.
2. The leaf samples were grinded in mortal with the pestle in 1 ml CTAB buffer (liquid nitrogen not used).
3. The solution is then transfered to 2 ml eppendorf tubes and incubated in water bath at 65-70°C for 1 hr.
4. The solution was allow to attain room temperature, equal volume of chloroform isoamyl alcohol 24:1 (equal volume to CTAB buffer) was added to it and centrifuged at 12,000 g for 10 minutes.
5. The supernatant was transfered carefully in fresh 2 ml eppendorf tube & remaining were discarded.
6. The ice cold isopropyl alcohol (2/3) volume of the supernatant was added in the tube. (Invert slowly thrice to precipitate DNA, small fiber of DNA sitting down was observed) and kept for incubation at 4°C for overnight.
7. It was then allowed to room temperature and centrifuged at 12,000 g for 10 minutes, supernatant discarded and pellet was collected.
8. Tubes containing pellet were allowed to air dry for 5–10 minutes and inverted on tissue paper to complete run off any supernatant.
9. The DNA pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 g for 10 minutes.
10. Later 70% alcohol discarded from tubes and allowed to air dry for 15 min on tissue paper in inverted position. Pellet was dissolved in 100 ul NFW and stored in -20°C for further downstream procedures. The DNA quantity for each sample was assessed on 0.8% agarose gel.

PCR amplification

The amplification of DNA from the samples were carried out by using Polymerase Chain Reaction. The PCR reaction mixture content are as 100ng of DNA dissolved in 2µl was used as template in 20µl of reaction mixture. The dNTP mix 2µl, 10X PCR buffer(with $MgCl_2$) 2µl, primers (F/R) 2µl, Taq DNA polymerase 1µl and total volume is makeup with nuclease free water 11µl. The vial containing all this PCR mix is set in PCR program shown in table 2.

Table 2. Shows the PCR programme used for DNA amplification using SSR markers

Sr no	Step	Temperature	Time	Cycle
1	Initial denaturation	95°C	5min	0
	Denaturation	94°C	20 sec	5
	Annealing	51°C	20 sec	
	Extension	72°C.	30 sec	
2	Denaturation	94°C	20 sec	35
	Annealing	56°C	20 sec	
	Extension	72°C	30 sec	
3	Final extension	72°C	20 min	

Gel electrophoresis of PCR products

The amplified PCR products were mixed with DNA loading dye and loaded on 4% agarose gel stained with EtBr (ethidium bromide) and electrophoresis run was set at constant 200 V for 3 hours. After complete run, gel was visualised under UV light in gel documentation unit for visual examination of SSR-specific markers DNA from Pegionpea.

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3. Results

The DNA and SSR-specific markers amplified DNA fragments from Pegionpea test samples are used for spectrophotometer and other techniques for analysis.

3.1 DNA quality and quantity

The concentration of DNA was determined by Nano Photometer, in which 1µl of dissolved DNA of test samples was considered. Sample 1: absorbance ratio is 1.671 and concentration is 2.273µg/ml, sample 2: absorbance ratio is 1.543 and concentration is 2.455 µg/ml, sample 3: absorbance ratio is 1.183 and concentration is 2.421µg/ml. The figure 1 shows DNA from Pegionpea samples, hybrid and parental lines.

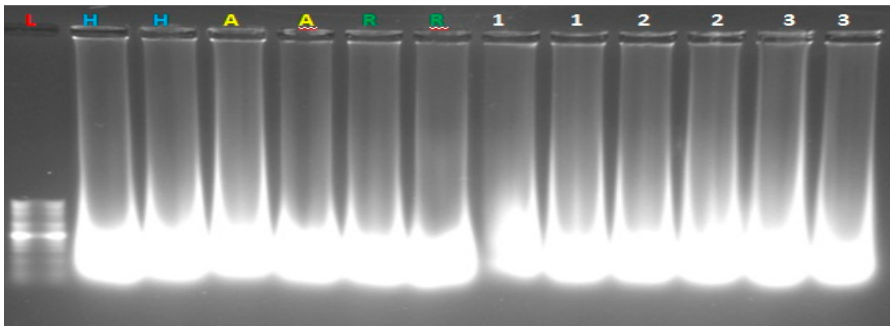
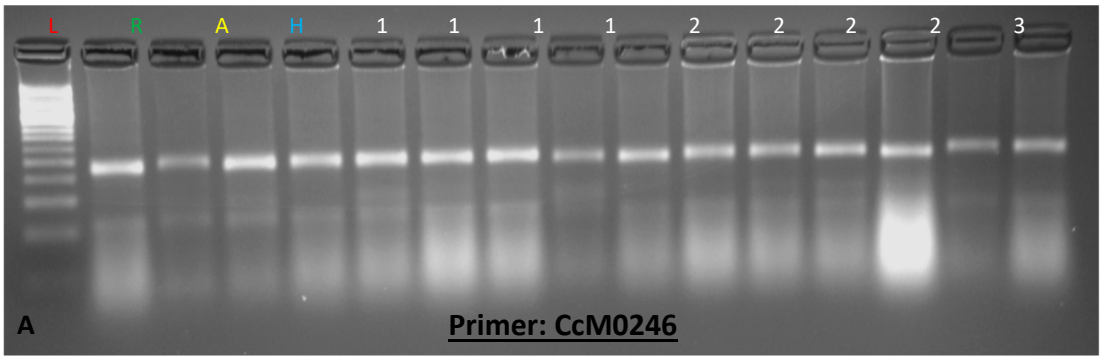


Figure1. Shows DNA extracted from Tur leaf were as L : Ladder (100bp), R : R Line (Male), A : A Line (Female), H : Hybrid, sample 1: Lot no. AP-DSR-1609-16H, sample 2: Lot no. 01-P39-08-03-07 and sample 3: Lot no. 01-P39-08-03-11.

3.2 PCR Amplification of SSR-markers

The DNA of hybrid, parent and test samples were used for SSR-marker specific amplification. In all seven SSR-primers were used against sample 1, 2, 3, however only three parents primers were amplified successfully. The amplification of DNA carried out by Polymerase Chain Reaction from test samples uses a programme given in table 2. Briefly 2µl of dissolved amplified DNA was used to check the fingerprinting along with marker ladder. Figure 2 shows SSR profiling for sample 1, 2, 3 each using SSR-primer CcM0246, CcM0516 and CcM0133.



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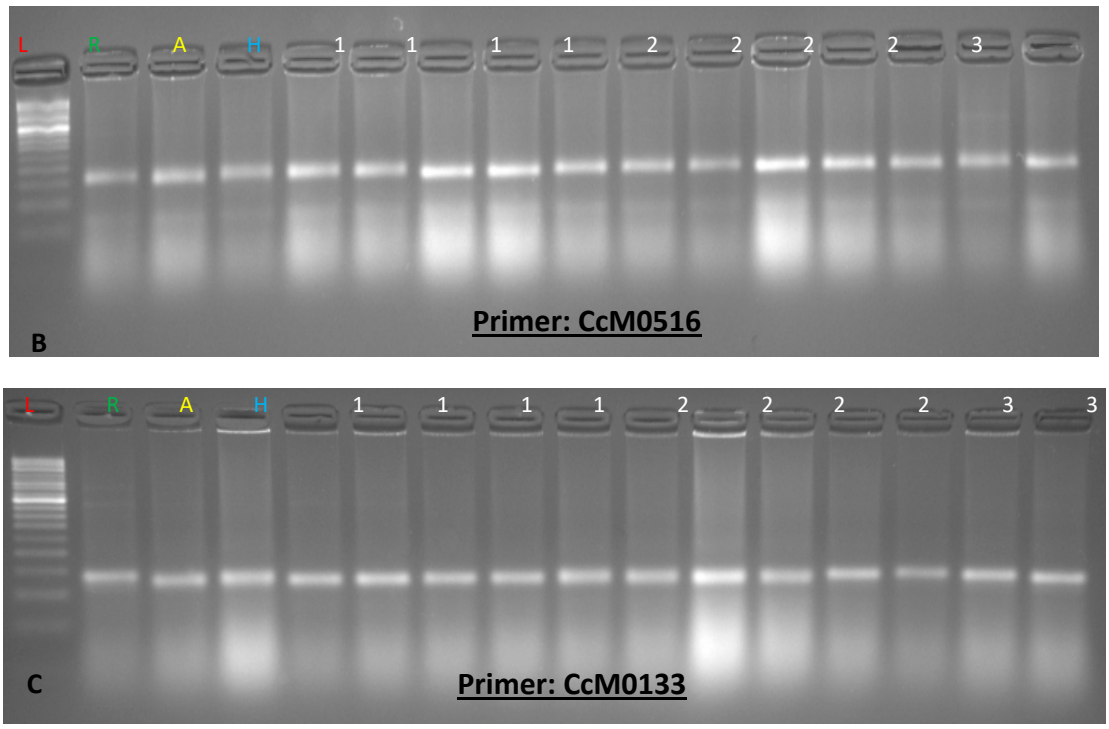

Figure 2. SSR profiling in 4% Agarose gel used for four seedling sample 1, 2, 3 each using SSR primer CcM0246, CcM0516 and CcM0133. H stands for hybrid, A and R as parental lines used as reference, L is 50bp ladder.

3.3 Allele scoring and analysis

The allelic data was analysed from seven primers out of which 3 SSR-primers shows amplification in sample 1, 2, 3. Yet primer CcM0246 confirms hybrid seed in sample 1 with two separate alleles. The allelic data was analysed after electrophoresis and DNA fingerprinting from sample 1. It was observed that only CcM0246 marker was able to amplify two different loci. (see figure 3) That shows hybrid with two alleles (bands) similar in test sample 1.

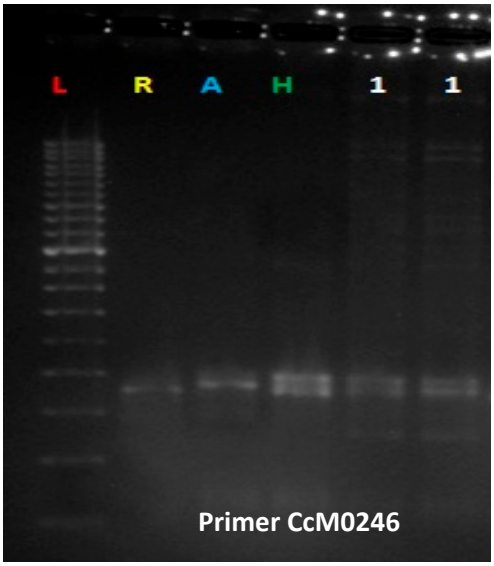

Figure 3. The DNA profiling of SSR primer CcM0246 in test sample 1.

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4. Discussion

The concentration of DNA in 1µl of DNA of test samples, sample 1,2,3 is 2.273µg/ml, 2.455 µg/ml and 2.421µg/ml respectively, which selective states that the adopted protocol is good for DNA isolation from Pegionpea. The PCR products were checked on 1.2% agarose gel but did not showed clear bands instead of that 3% and 4% gel were used for optimisation and good clarity of bands was found on 4% gel.

In total seven primers were consider for experimentation, nevertheless only three were success in amplification of DNA from Pegionpea samples. Three primer pairs (CcM0246, CcM0516 and CcM0133) which showed polymorphism in three samples. The primer CcM0246 should produced two distinct alleles of different sizes, one at 251bp and the second at 245 bp. Similarly primer CcM0516 should produced two distinct alleles of different sizes, one at 207 bp and the second at 211 bp. Consequently, primer CcM0133 should produced two distinct alleles of different sizes, one at 200 bp and the second at 208bp.

The designed SSR-primers provided by ICRISAT have 4-8bp difference which becomes hurdle for DNA profiling interpretation. Thus, the SSR-primers that produced nore bp differences may solve this drawback. The work of [1] designed SSR-primers and have modified the Tm accordingly on basis of BES sequence obtained from NCBI and have used them successfully in their studies.

The hybrid must produce two alleles of different sizes specific to each SSR-primers (see figure 2). Among the test samples 1,2,3 the PCR amplification of marker CcM0246 produced heterozygous alleles only in samples 1. Thus it is confirmed that marker CcM0246 is highly heterozygous to produced two different alleles and these often differed between individuals. The [4] have designed thirty-five SSR-primers that showed polymorphism among 24 pigeonpea breeding lines. In our work other markers CcM0516 and CcM0133 could not produce two different loci was therefore this marker not useful for purity and hybrid testing in this germplasm.

The SSR-primers used in present studies Primer CcM0246 is useful as it has shown similar bands in sample 1 to that of reference hybrid, whereas sample 2 and 3 could not give the confirmation of similarity to that of reference hybrid. Similarly, Primer CcM0516 showed polymorphism but none of the sample gave the confirmation of similarity to the reference hybrid. In contrast, Primer CcM0133 showed similar bands in sample 2 to that of reference hybrid. The sample 1 and 3 cannot give the confirmation of similarity. The band observed in sample 2 have similar intensity, width with hybrid. But, the clear band is not visible which become a cause to labelled it unclear results. The table 3 shows the characteristics of SSR primers. The SSR-markers are extensively used in hybrid testing, genetic mapping and diversity. Recently, [5] demonstrated utility of SSR markers in trait mapping through association and bi-parental linkage analyses.

Table 3. Shows the characteristics of SSR primers observed in present work.

Name of Primer	Reflection of base pair size(bp)			Remarks
	A line (F)	R line (M)	Hybrid	
CcM0246	251	245	251, 245	Shows two distinct bands observed in hybrid and sample 1
CcM0516	207	211	207, 211	Shows single bands in hybrid and in test samples
CcM0133	200	208	200, 208	Shows single bands in hybrid and in test samples

5. Conclusions

This study investigated the hybrid testing in pigeonpea test varieties with SSR markers. The high quality DNA was obtained successfully from leaves samples using adopted protocol. The PCR products were run in optimized electrophorosis of 4% gel to produce good separation of DNA. Out of seven three SSR-primers were successful to produce DNA fingerprints. The primer CcM0246 shows clear separate alleles in test sample 1. Therefore primer CcM0246 may be useful to check the purity in sample 1. The primer CcM0133 shows DNA bands similar to reference hybrid but could not give clear explanation for its purity. Further studies should exploit with more new SSR markers using sequenced pigeonpea genomes. Also validate primers of large base pair differences between parental lines could help to identify the purity of hybrid and pigeonpea samples.

Author Contributions: The Conceptualization, DL and BL; Methodology, DL and BL; Formal Analysis, DL and BL; Investigation, DL; Writing-Original Draft Preparation, DL and BL; Writing-Review & Editing, DL and BL.

Funding: DL have worked as Junior Research Analysis in seed testing lab, This research received no external funding.

Acknowledgments: Authors are thankful to DNA Fingerprinting Lab, Seed Testing Laboratory, Nagpur, for providing research facility to conduct experimentations. Authors are thankful to Tina Puttawar (Agri. Asst.), Goutam Moon (Seed Testing Officer) and Shyam Gharpure (Agri. Supervisor), Seed Testing Laboratory Nagpur for providing samples, allowing to perform experimentation, suggestions and discussion for improving manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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