Hybrid Testing In Pigeonpea Using DNA Fingerprinting By SSR-Markers
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Abstract: Pigeonpea (Cajanus cajan (L.) is a drought tolerant crop and one of the most important legumes grown in the Tropic and sub tropic region, popularly known as arhar dal (tur dal). It is largely used as supplement cereal because it is a rich source of protein for humans.

INTRODUCTION
Pigeonpea, Cajanus cajan [L.] is a drought tolerant crop and one of the most important legumes grown in the Tropic and sub tropic region, popularly known as arhar dal (tur dal). It is largely used as supplement cereal because it is a rich source of protein for humans.
Which were used for DNA extraction, PCR experimentation.

Reagents and chemicals

The reagents and chemicals used for preparation of CTAB buffer (100 ml) are as follows:

- 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 H2O 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 (see table 1), TAE buffer, agarose gel and ethidium bromide [3].

SSR-marker Primers

The SSR primers used in the present study were obtained from ICRISAT and details of SSR-markers such as sequences, melting temperature 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.

| Sn | Primer name | Sequences (5’ 3’) | Length (bp) | Tm | Expected amplified bands |
|----|-------------|-------------------|-------------|----|-------------------------|
| 1  | CcM0246     | ATGGAGCCAAAAGTGTCCAAG (F) | 20 | 52°C | 251bp (A) |
|    |             | ATTTAGGTGTTGTTGGACAGA (R) | 20 | 50°C | 245bp (R) |
| 2  | CcM0516     | ATTTAGGTGTTGTTGGACAGA(F) | 20 | 50°C | 207 bp (A) |
|    |             | TTTGTCACCTACTTGTTACC (R) | 20 | 54°C | 211 bp (R) |
| 3  | CcM0008     | CCGTGAAAAAGGTTCAATGAG (F) | 20 | 52°C | 208 bp (A) |
|    |             | CAAAATAAAGCCTACTTGTTTACGA (R) | 27 | 51°C | 202 bp (R) |
| 4  | CcM0207     | TTTTGCGGCTCATTTAACC (F) | 20 | 48°C | 256 bp (A) |
|    |             | TAAATGGGAGCAACACTGTA (R) | 20 | 52°C | 254 bp (R) |
| 5  | CcM0494     | AGCTGAAAAATCCGCAACTT (F) | 20 | 48°C | 129 bp (A) |
|    |             | GTCTGTGTGTCAAAATCCAACTT (R) | 23 | 50°C | 138 bp (R) |
| 6  | CcM0133     | GTTGTCCATTTTGAACCTCC (F) | 20 | 52°C | 200 bp (A) |
|    |             | CCATAATCCCAAATCCAAATCCA (R) | 21 | 49°C | 208 bp (R) |
| 7  | CcM0948     | GCACAGGTCAAGTCTGTACC (F) | 20 | 56°C | 235 bp (A) |
|    |             | CATTTTCCCACCTTTCTTGTA (R) | 20 | 50°C | 239 bp (R) |

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:

- Fresh 100-500 mg of young leaf samples of pigeonpea were taken.
- The leaf samples were grinded in mortal with the pestle in 1 ml CTAB buffer (liquid nitrogen not used).
- The solution is then transfered to 2 ml eppendorf tubes and incubated in water bath at 65-70°C for 1 hr.
- The solution was allowed 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.
- The supernatant was transferred carefully in fresh 2 ml eppendorf tube & remaining were discarded.
- The ice cold isopropanol 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.

- It was then allowed to room temperature and centrifuged at 12,000 g for 10 minutes, supernatant discarded and pellet was collected.
- Tubes containing pellet were allowed to air dry for 5–10 minutes and inverted on tissue paper to complete run off any supernatant.
- The DNA pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 g for 10 minutes.
- 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 MgCl2) 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 amplified DNA from Pegionpea.

RESULTS

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

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 2.273 µg/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 2.421 µg/ml. The figure 1 shows DNA from Pegionpea samples, hybrid and parental lines.

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 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.
Fig-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.

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.

Fig-3: The DNA profiling of SSR primer CcM0246 in test sample 1.
DISCUSSION

The concentration of 1µl DNA of test samples 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. Even only one marker is showing hybridity, besides the two bands observed in the hybrid does not seem exactly the same as amplified in respective parental lines. Thus, the SSR-primers that produced more 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 therefore these markers are not useful for purity and hybrid testing in this germplasm.

The SSR-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...
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.

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