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Assessment of Molecular Diversity in Popular Pigeonpea Genotypes using Genic Microsatellite Markers

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Fusarium wilt is an important soil borne disease of pigeonpea causing significant yield losses in susceptible cultivars throughout the pigeonpea growing areas. Thirty one diverse pigeonpea genotypes were screened for wilt resistance and analyzed using 14 genic SSRs which included five SSR markers that were reported to differentiate resistant and susceptible genotypes. Polymorphic information content ranged from 0.69 to 0.90 and SSR marker ASSR-352 displayed the highest PIC value of 0.90. Dissimilarity coefficient ranged from 0.4 to 0.93. Earlier studies have identified ASSR 352 as one of the highly polymorphic genic SSRs in pigeonpea. The marker is associated with

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major intrinsic protein.Highest similarity was observed between genotypes LRG 133-33 and TDRG 59/ICPL 99050. The 14 SSR markers generated 116 alleles and the genotypes were grouped into 4 clusters. Cluster 3 is the largest with 10 genotypes and comprises of two subclusters of wilt resistant genotypes and one subcluster of susceptible genotypes. Subcluster 2 consists of resistant and moderately resistant genotypes except PRG 176 & WRGE 93. The SSR markers used in our study could differentiate the resistant and susceptible genotypes to some extent.

Keywords: SSR; Fusarium wilt; genetic diversity; pigeonpea; genic SSRs.

1. INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is one of the major pulse crops of India. India is the largest producer and consumer of pigeonpea in the world with 70% of the production. It plays a key role in food security, balanced diet and subsistence agriculture through integrated farming systems and symbiotic nitrogen fixation Analyzing genetic relationships and studying variability is important for revealing diversity available in the species, to conduct successful breeding programs and also for germplasm conservation [1]. Among the molecular markers many studies have reported the potential of SSR markers for studying population structure and genetic diversity because of high polymorphism, detection of multi-allelic variation, co-dominance, reproducibility and ease of detection [2, 3].

Fusarium wilt and sterility mosaic disease are the two major biotic constraints affecting pigeonpea production. Pigeonpea wilt caused by a fungus *Fusarium udum* Butler is soil borne resulting in yield losses upto the extent of 100% if the disease incidence occurs during flowering stage of crop growth [4]. Being soil borne disease, the use of fungicides is not the effective way to control the disease and development of resistant varieties is the most viable option. Four different races of *Fusarium* pathogen have been reported with pathogenic race present in Bangalore being most virulent. Wilt symptoms usually appear during flowering and podding stage of the crop. Fusarium wilt resistance in pigeonpea is controlled by different gene actions depending on the genetic backgrounds, ranging from single to multiple genes (complementary to duplicate gene action) [5]. Wild relatives and land races play an important role in the genetic improvement of cultivated crops especially tolerance to biotic and abiotic stresses. *Cajanus scarabaeoides* (L.) Thouars is one of the important wild species, which possess several desirable traits [6].

Resistant sources for wilt disease are available, but screening requires the development and maintenance of wilt sick plot. This is not only laborious but also time consuming as it takes one entire crop season. The studies conducted by various workers suggested the presence of significant marker traits association for *Fusarium* wilt of pigeonpea and [7-9]. However closely linked markers associated with wilt disease or workable markers are not available to carry out marker assisted breeding. Molecular markers for differentiation of resistant and susceptible genotypes can aid the breeders in the selection of parental lines. Thirty-six elite pigeonpea genotypes adapted to different agro climatic regions have been grouped into two main clusters based on wilt reaction and six genic SSR markers were reported to be significantly associated with *Fusarium* wilt resistance [8]. The inheritance of resistance to *Fusarium* wilt disease in long duration pigeonpea genotypes is governed by one dominant gene in BDN-2004-1 and BDN-2001-9, two duplicate dominant genes in BWR-133 and two dominant complimentary genes in resistance source IPA-234. The group validated the association of SSR markers ASSR-1, ASSR-23 and ASSR-148 with wilt for use in pre-screening of diverse parental lines [7]. The present study was carried out to assess genetic diversity in a set of elite pigeonpea genotypes and evaluate the potential association of recently reported SSR markers with Fusarium wilt disease resistance.

2. MATERIALS AND METHODS

2.1 Plant Material

A set of thirty one pigeonpea genotypes including released varieties, germplasm and advanced breeding lines were selected for this study. The genotypes included both wilt resistant and susceptible genotypes which were grown in pots and leaves from 20 days old seedlings were collected and frozen in liquid nitrogen (Table 1).

Table 1. List of Pigeonpea genotypes used for diversity analysis along with Fusarium wilt reaction

S.No	Genotype	FW reaction
1.	PRILB130	R
2.	ICPL20096	R
3.	PRILC 138	R
4.	ICP 7035	R
5.	ICP11015	R
6.	ICP8860	S
7.	ICPL9045	S
8.	PRILC 54	S
9.	PRILC 9	S
10.	LRG 30	S
11.	TDRG 58	R
12.	BSMR 736	R
13.	ICP7119	S
14.	LRG 133-33	S
15.	TDRG 59/ICPL 99050	R
16.	CRG 2012-25	R
17.	RVSA 16-1	S
18.	CRG 150	S
19.	AGL 1640	R
20.	ICPL 161	S
21.	WRGE 122	S
22.	ICP11230	S
23.	ICPL 2376	S
24.	WRGE 93	S
25.	TS 3R	R
26.	WRGE 121	MR
27.	PRG 176	S
28.	TDRG-4	R
29.	TDRG 107	R
30.	WRGE 120	MR
31.	ICPL 87119	R

R: Resistant; MR: Moderately Resistant; S: Suceptible

2.2 Genomic DNA Isolation and PCR using SSR Markers

200mg of leaf tissue was ground into fine powder and genomic DNA was extracted following CTAB procedure [10]. The quality and quantity of DNA was checked through agarose gel electrophoresis (0.8%). A total of 14 ASSR primers were used for PCR amplification (Table 2). The PCR mixture comprised of 25ng of genomic DNA, 2x EmeraldAmp GT PCR Master Mix, 2.5pm of forward and reverse primers. Amplification was carried out in thermal cycler (Master Cycler, Eppendorf) by initial denaturation at 94^0C for 5 min followed by 35 cycles comprising of denaturation at 94^0C for 1min, annealing temperature of 55^0C for 30 sec, primer extension at 72^0C for 30 sec and final extension of 7 min. The PCR products were resolved using 3% metaphor agarose and documented in gel documentation system.

2.3 Statistical Analysis

For genetic diversity studies the PCR products from SSR were analyzed by scoring qualitatively for presence (1) or absence (0). A genetic dissimilarity between the cultivars was measured by the dissimilarity coefficient. Polymorphism Information Content (PIC) was calculated according to using the following equation [11]:

$$PIC_{j=1} - \sum^{n}_{i=1} Pi^2$$

Where, i = the i^{th} allele of the j^{th} marker, n = the number of alleles at the j^{th} marker and P = allele frequency. Hierarchical cluster analysis was performed using software DARWin ver 6.0.2

Table 2. List of polymorphic SSR markers along with primer information

S.No	Primer Code	Forward primer sequence	Reverse primer sequence	Motif
1	ASSR 277	GGAAATACAAATAGCGTAGTGG	CAGCCTAGAAACTTAACACGAG	$(TCCTGT)_5$
2	ASSR 304	ACTCAACGGTGCTACTCATC	AACCGATGCTTTCTTCTACTAC	$(GTT)_7$
3	ASSR 66	CAAGTAAACGATTCCTCTTCTC	GTTCCCTATAGTTGATGTTTGG	$(CT)_{12}$
4	ASSR 148	AACCGATGCTTTCTTCTACTAC	ACTCAACGGTGCTACTCATC	$(CAA)_7$
5	ASSR 352	CACAAACCTGTAACTTGATCC	GTGACGAGGATATACAAATTCC	$(TTTAA)_6$
6	ASSR 97	GAGGATGAAGAGGAAGAGTATG	TAGCCATCATCTTGTTATGCTG	$(ATGGAC)_8$
7	ASSR366	CTCTGCAACTCGCTCATTTC	ACGTGATGGAGAAGATCCAAC	$(CGT)_8$
8	ASSR 1	GTCCGTTGAAAAACAAAGAG	CGTTTTAGGTTTCTTCTCTGC	$(GGA)_{10}$
9	ASSR 229	ATAGTGGGACAGTAGAAAATCC	CAACTCATCTCTTGGTTCTCC	$(TAAGGG)_5$
10	ASSR 23	CTTTCCCTTCTCTCTCAACAC	AAGCAGAAGCAGAAGCAGAG	$(CCTTCT)_5$
11	ASSR 363	GGGAGAAGTATAAGGAGAAATG	TCACCCTTTGATAATGTTCC	$(GCATCA)_5$
12	ASSR 70	TGTTAACTGTAGTGGGAGTGAG	AGAGACTACCGTAAACGAACAC	$(GGTAGA)_6$
13	ASSR 317	AATCAGTGACAGTGAGGAAAAG	ACGTAACCTCCTCCTCGTTTAC	$(GAGCAT)_9$
14	ASSR 228	TAGAGGAGGTGAAAAAGGATAG	TTTACTTTACCCGTACCCTTAC	$(CTAAGG)_5$

[12] for calculating genetic relationship among individuals, allelic data was used to generate pair wise dissimilarities using simple matching coefficients. The dissimilarity coefficients were used to construct neighbor-joining tree.

3. RESULTS AND DISCUSSION

3.1 Polymorphic Information Content (PIC) of SSR Markers

A set of thirty one pigeonpea genotypes comprising of germplasm, advanced breeding lines and released cultivars from different states and ICRISAT were included in this study. The genotypes included wilt resistant, moderately resistant and susceptible cultivars (Annual reports, PJTSAU). Fourteen genic-SSR markers were used to analyze genetic diversity among the pigeonpea genotypes [13, 8]. These included the six markers (ASSR-1, ASSR-23, ASSR-148, ASSR-229, ASSR-363 and ASSR-366) that were reported to have significant association with *Fusarium* wilt resistance [8]. The 14 SSR markers revealed 116 alleles with an average of 8.2 alleles. PIC ranged from 0.69 to 0.90 indicating the usefulness of the markers used in the study. The average PIC value for all the 14 markers was 0.82, while SSR marker ASSR-352 displayed the highest PIC value of 0.90 (Table 3, Fig. 1). The size of the amplified products ranged from 110-272 bp. Earlier studies identified a total of 83 putative alleles from the 16 polymorphic microsatellite markers with PIC ranging from 0.11 to 0.80 [14]. While some studies reported very low level of polymorphism in pigeonpea varieties with SSR markers [3]. Molecular characterization of five distinct pigeonpea genotypes as carried out using antioxidant (AO) enzyme [*APX* and *SOD*] and pathogenesis-related (PR) protein [*CHS* and β-*1, 3-glucanase*] families [15].

3.2 Estimation of Genetic Similarities

Several studies have reported low level of molecular diversity in pigeonpea gene pool using various marker systems such as AFLP [16] DArT [17] and SSRs [18]. In this study genetic dissimilarity matrices of all twenty five genotypes were generated using genic SSR markers (Darwin version 6.0) [12]. The genotypes were classified as susceptible/resistant/moderately resistant based on wilt screening at ICRISAT/ARS, Tandur locations (Annual reports of AICRP on pigeonpea). Allelic diversity data was used to generate dendrogram explaining the genetic relationships among the genotypes. The genetic dissimilarity coefficient between individuals based on SSR data ranged from 0.40 to 0.95. Minimum dissimilarity coefficient of 0.4 was observed between genotypes LRG 133-33 and TDRG-59 of cluster IV. Similarly genotypes RVSA 16-1 and CRG 150 of cluster I also displayed minimum dissimilarity coefficient of 0.4. Maximum dissimilarity coefficient of 0.95 was observed between the wilt resistant genotypes TDRG 107 and PRILB-130 which belong to clusters II and III. Total soluble proteins can be employed as biochemical markers to assess the genetic variability in pigeonpea. Analysis of total soluble seed protein profiles classified the pigeonpea wild species into two

Table 3. Allelic information and Polymorphic information content of SSR markers

S.No	Primer Code	No. of alleles	Allele size range (bp)	PIC
1	ASSR 277	8	134-150	0.83
2	ASSR 304	5	123-132	0.73
3	ASSR 66	9	191-216	0.85
4	ASSR 148	7	125-138	0.83
5	ASSR 352	9	138-162	0.9
6	ASSR 97	8	144-175	0.88
7	ASSR 366	11	230-272	0.89
8	ASSR 1	8	110-121	0.89
9	ASSR 229	10	154-178	0.86
10	ASSR 23	12	149-175	0.86
11	ASSR 363	9	164-188	0.88
12	ASSR 70	7	167-185	0.70
13	ASSR 317	8	160-178	0.78
14	ASSR 228	5	138-149	0.69

major clusters with 6 out of 7 wilt resistant accessions in one cluster [6]. Genetic diversity in pigeonpea A and R lines was estimated using 123 SSRs and 54.47% polymorphism was reported [19]. Genetic divergence in ninety six accessions of pigeonpea including 15 male sterile, 13 maintainer and 68 germplasm were analyzed using 33 polymorphic primers. Genotypes were grouped into 8 clusters with highest dissimilarity coefficient (32%) between Pusa 991 and ULA 11 [20].

3.3 Clustering of Pigeonpea Gentoypes

The fourteen polymorphic primers grouped the 31 genotypes into 4 major clusters with cluster III having maximum number of genotypes (Fig. 2).

Custer I consisted of two sub clusters: cluster IA with wilt susceptible genotypes, cluster IB comprised of all wilt resistant and susceptible genotypes. Cluster II consisted of all wilt resistant genotypes except PRG 176 & WRGE 93. Pigeonpea entries developed at RARS, Warangal (WRGE 93, WRGE 121, and WRGE 120) were grouped together in cluster II indicating higher genetic similarities. Cluster III comprised of ten genotypes which were further classified into three distinct sub clusters. The SSR markers could clearly differentiate the wilt susceptible and resistant genotypes. The susceptible genotypes were grouped into IIIA while IIIB and IIIC comprised of wilt resistant genotypes. Cluster IV comprised of all wilt susceptible genotypes except TDRG 59 & CRG 2012-25 (Table 4).

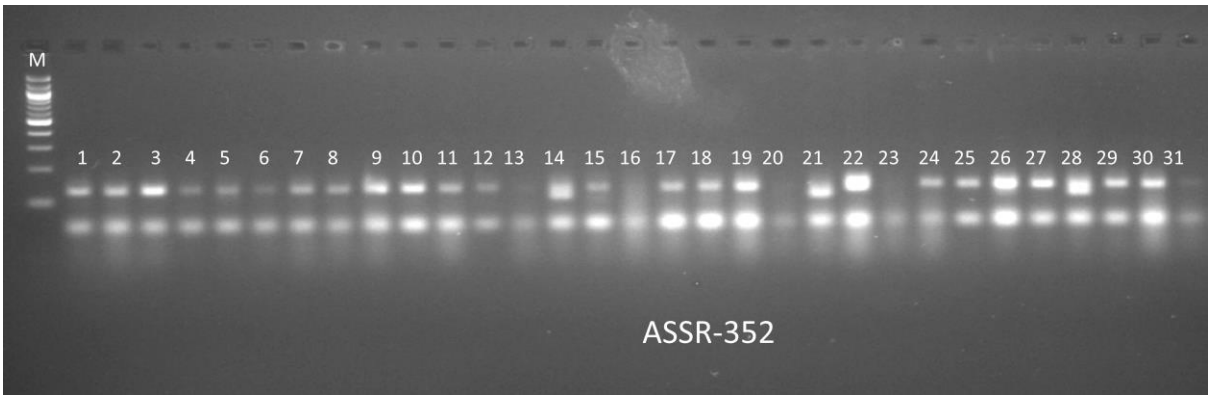

Fig. 1. Gel image depicting allelic variation in pigeonpea genotypes with ASSR-352

M= 100bp DNA ladder; Lanes 1-32 pigeonpea genotypes: 1. PRILB130, 2. ICPL20096, 3. PRILC 138, 4. ICP 7035, 5. ICP11015, 6. ICP8860, 7. ICPL9045, 8. PRILC 54, 9. PRILC 9, 10. LRG 30, 11. TDRG 58, 12. BSMR 736, 13. ICP7119, 14. LRG 133-33, 15. TDRG 59/ICPL 99050, 16. CRG 2012-25, 17. RVSA 16-1, 18. CRG 150, 19. AGL 1640, 20. ICPL 161, 21. WRGE 122, 22. ICP11230, 23. ICPL 2376, 24. WRGE 93, 25. TS 3R, 26. WRGE 121, 27. PRG 176, 28. TDRG-4, 29. TDRG 107, 30. WRGE 120, 31. ICPL 87119

Table 4. Clustering of pigeonpea genotypes based on SSR markers

Clusters	Genotypes
Cluster 1	ICPL 161, ICP11230, ICPL 2376, WRGE 122 (all susceptible) RVSA 16-1, CRG 150, AGL 1640, BSMR 786
Cluster 2	WRGE 93, WRGE 121, WRGE 120, PRG-176, TS3R, TDRG 4, TDRG 107, ICPL 87119 (resistant & moderately resistant except PRG 176 & WRGE 93)
Cluster 3	ICP8860, ICPL 9045, PRIL C 54, PRILC 9 (all susceptible) PRILB130, ICPL 20096, PRIL C 138 (all resistant) ICP 7035, ICP11015, TDRG 58 (all resistant)
Cluster 4	ICP 7119, LRG 133-33, TDRG 59/ICPL 99050, CORG 2012-25, LRG 30

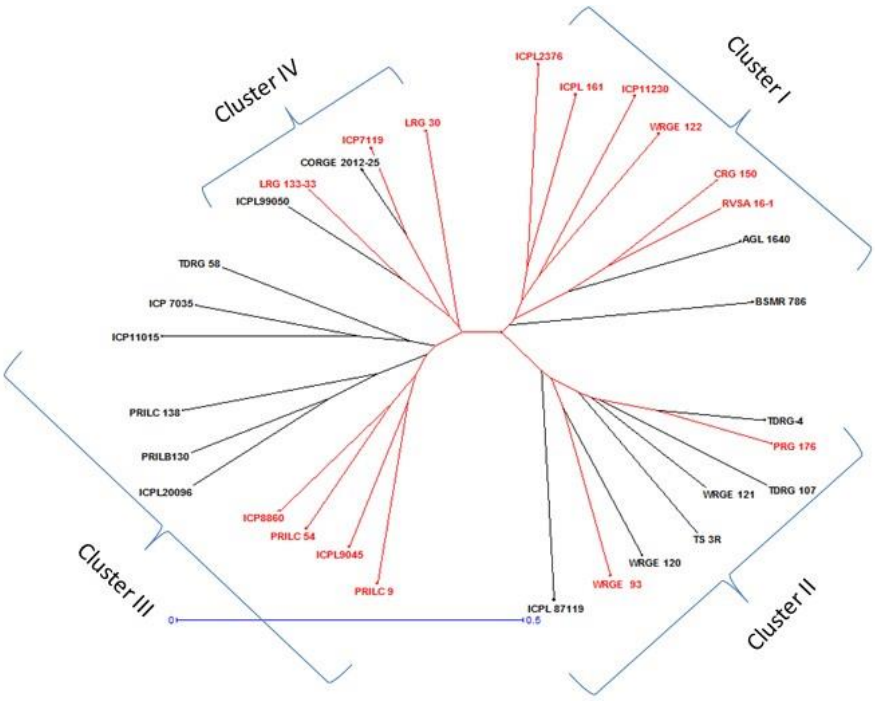

Fig. 2. Weighted neighbor-joining dendrogram of pigeonpea genotypes depicting four major clusters. Color key: Susceptible entries (Red)

The SSR markers used in this study could differentiate the wilt susceptible and resistant genotypes to a major extent indicating their usefulness in the selection of parental lines for breeding program. Principle component analysis (PCA) also, classified the pigeonpea genotypes into 4 distinct clusters. The grouping of the genotypes remained almost the same except for genotypes LRG 30 and LRG-133-33 of cluster IV which were shifted to cluster III. Molecular marker correlated with Fusarium wilt will aid the breeders in selection of parental lines for resistance breeding in African and Asian Pigeonpeas [21]. Recent studies have reported SNPs/Indels for Fusarium wilt and sterility mosaic diseases resistance in pigeonpea [9]. Five SSR markers from earlier studies [8], ASSR 363, ASSR 366, ASSR 1, ASSR 23 and ASSR 148 could clearly differentiate a set of 19 wilt resistant and susceptible genotypes with marker ASSR 363 having highest PIC [22, 23].

4. CONCLUSION

Genetic diversity in 31 elite pigeonpea Fusarium wilt resistant and susceptible genotypes was analyzed using 14 genic-SSR markers. ASSR-352 is the most useful marker in discerning genetic diversity. Fusarium wilt resistant genotypes of divergent clusters are useful in selection of parental lines hybridization program and also for mapping of wilt resistance in pigeonpea.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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