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Genetic Characterization of Recently Developed Germplasm and Validation of SSR Markers Linked to Fusarium Wilt and Pigeonpea Sterility Mosaic Disease in Pigeonpea [*Cajanus cajan* (L.) Millsp.]

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ABSTRACT

Background: Pigeonpea is an important grain legume crop and a source of vegetarian protein. To meet the growing food demand, there is a pressing need to develop high-yielding disease-resistant varieties of pigeonpea. Hence, the present study was conducted to characterize the recently developed genetic material for yield and yield-attributing traits, identify the resistant sources and validate the SSR markers linked to fusarium wilt (FW) and sterility mosaic disease (SMD) resistance in pigeonpea.

Methods: Pigeonpea genotypes were evaluated in two environments for yield and yield-related traits to assess the genetic variability parameters. The genotypes were phenotypically screened for their response to fusarium wilt and pigeonpea sterility mosaic disease under controlled conditions.

Result: High PCV, GCV, heritability and genetic advance as per cent of mean values were obtained for the traits number of primary branches plant^{-1}, number of secondary branches plant^{-1}, number of pods plant^{-1}, pod bearing length and seed yield plant^{-1} in both environments. The diversity analysis using *k*-means clustering grouped the genotypes into nine clusters with high inter-cluster distance between clusters I and VIII indicating high genetic diversity between the lines present in these two clusters. The six genotypes (ICPL 15023, ICPL 15063, ICPL 19467, ICPL 19482, ICPL 19489 and ICPL 19499) showed a combined resistance response. The validation of reported linked SSR markers was done using 52 resistant and susceptible germplasm lines. Four markers, AHSSR 50, AHSSR 34, AHSSR 20 and CcM0588 and seven markers *viz*., ASSR 23, ASSR 229, ASSR 363, HASSR 18, HASSR 121, HASSR 128 and CcGM 03681, were able to differentiate resistant and susceptible genotypes for SMD and FW, respectively. Subsequently, one marker (AHSSR 20) for SMD and four markers (ASSR 229, HASSR 121, HASSR 128, CcGM 03681) for FW were found significantly associated with disease resistance based on single marker analysis based on t-test. The identified potential genotypes can be used as a source of resistance to SMD and FW or directly released for commercial cultivation after extensive testing.

Key words: Fusarium wilt, Genetic variability, Inter-cluster distance, *K*-means clustering, Pigeonpea, Resistance, SSR markers, Sterility mosaic disease.

INTRODUCTION

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an important multipurpose grain legume crop with a chromosome number of 2n = 22 and a genome size of 833 Mb (Dutta *et al.,* 2011). Pigeonpea is native to India and is widely grown in the tropics and subtropics. India is the world's top producer of pigeonpea, followed by Africa, Australia, Malawi, Tanzania, Kenya and Uganda (Sarkar *et al.,* 2020). To meet the growing food demand, breeding for high yield remains the major breeding objective in crop improvement. In this context, the development and characterization of improved varieties that perform well under various agro-climatic conditions is crucial. It mainly depends upon the amount of genetic variability that exists in the genetic material for the traits considered (Xie *et al.,* 2015). Understanding the genetic architecture of yield and related traits is crucial for selecting appropriate breeding strategies to intensify crop improvement programs (Yerimani *et al.,* 2013; Saroj *et al.,* 2013). Furthermore, evaluating genetic diversity is essential for choosing diverse parentFs for hybridization programs.

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Genetic diversity assessment employs the non-hierarchical *k*-means clustering method, which categorizes variability into distinct 'k' clusters based on *k*-means algorithms (Macqueen, 1967). The *k*-means, a centroid or distance-based algorithm, assigns points to clusters by computing distances. It creates genetically diverse clusters based on genetic distances between genotypes, facilitating the identification of diverse genotypes (Kanavi *et al.,* 2020).

The high sensitivity of pigeonpea to many biotic and abiotic stresses has hindered the harnessing of its production potential. Major biotic stresses of pigeonpea include Sterility mosaic disease (SMD) and Fusarium wilt (FW) which cause a yield loss of up to US$113 million (Singh *et al.,* 2016). The sterility mosaic disease (SMD) also called "Green Plague", is caused by an Emaravirus *i.e*., Pigeonpea Sterility Mosaic Virus-*PPSMV* which is transmitted by eriophyid mite *Aceria cajani* Channabasavanna in a semi-persistent manner (Kulkarni *et al.,* 2002). This disease causes an estimated yield loss of about 26-97% if infection occurs in early stages and later infection causes sterility of plants (Kannaiyan *et al.,* 1984). Fusarium wilt is a soil and externally seed-borne fungal disease and caused by *Fusarium udum* Butler, that affects the plant from seedling to the pod-setting stage resulting in wilting of plants. FW disease is markedly increasing in pigeonpea-growing states and causes an estimated loss of 29.60 to 99.90%, with variant 2 of *F. udum* specific to Karnataka (Ravikumar *et al.,* 2022). Management of these diseases through conventional methods is difficult emphasizing the need for developing resistant varieties as a practical and cost-effective approach.

The development of resistant varieties relies on the precision mapping of multiple resistance genes using appropriate marker systems and identifying molecular markers that are closely linked to disease resistance. Despite the successful tagging of resistance to SMD and FW by various DNA marker systems in pigeonpea, their application in marker-assisted breeding remains limited (Halladakeri *et al.,* 2023). To ensure the wider utilization of molecular markers in marker-assisted breeding, it is imperative to validate them across multiple genetic backgrounds (Bipinraj *et al.,* 2011).

Thus, the present study was conducted to identify high-yielding pigeonpea lines from the recently developed germplasm carrying resistance and to validate reported linked SSR markers for FW and SMD.

MATERIALS AND METHODS

Assessment of genetic variability and genetic diversity

To assess the genetic variability and genetic diversity, a set of 78 newly developed pigeonpea germplasm obtained from the ICRISAT, Hyderabad, was evaluated in two different environments *viz*., June (normal planting, environment I) and August (late planting, environment II) in Augmented design with BRG3 and BRG5 as checks. The experiment was carried out during the rainy season of 2021 at the All India Coordinated Research Project (AICRP) on Pigeonpea, Zonal Agricultural Research Station (ZARS), University of Agricultural Sciences, GKVK, Bengaluru.

Observations were recorded on ten yield and yield-attributing traits namely, days to 50% flowering, plant height (cm), number of primary branches plant^{-1}, number of secondary branches plant^{-1}, number of pods plant^{-1}, number of seeds pod^{-1}, pod bearing length (cm), pod length (cm), test weight (g) and seed yield plant^{-1} (g). The data were analyzed using Augmented statistical analysis (Federer, 1956) to assess the significant differences among the genotypes. Due to unavailability of statistical package for performing pooled ANOVA for augmented design, adjusted means were derived from each environment. These blocks adjusted means from both environments were then averaged to obtain the pooled means. These pooled adjusted means were used for subsequent analysis. Descriptive statistics and genetic variability parameters, such as the phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) (Burton and Devane, 1953), heritability (H) (Hanson *et al.,* 1956) and genetic advance as per cent of mean (GAM) (Johnson *et al.,* 1955), were estimated for each environment using R software Version 4.1.3 with the package "*augmentedRCBD*."

Additionally, genetic diversity was assessed through a model-based *k*-means clustering approach (Macqueen, 1967) using R software Version 4.1.3 and the package "*factoextra*".

Screening for SMD and FW

Along with the above 78 genotypes, 24 additional lines received from ICRISAT, Hyderabad, were phenotyped for their reaction to SMD and FW. Artificial screening for SMD and FW was carried out separately during the rainy season of 2021 and *summer* 2022, using the leaf stapling technique (Nene *et al.,* 1981) and root dip technique (Pande *et al.,* 2012), respectively. BRG 3 was utilized as a resistant check for both diseases, while ICP 8863 and ICP2376 served as susceptible checks for SMD and FW, respectively. Further, the genotypes were classified into different disease response classes based on the scale provided by Singh *et al.* (2003) for SMD and Pande *et al.* (2012) for FW. The per cent disease incidence (PDI) was calculated at 15 days and seven days intervals till 60 days after planting for SMD and FW, respectively using the formula:

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

Subsequently, the third and fourth-degree statistics *viz*., skewness and kurtosis were estimated using R software Version 4.1.3 and the package "*moments*" and Shapiro-wilk's normality test to test the nature of frequency distribution using R software Version 4.1.3 and the package "*dplyr*."

Validation of reported linked SSR markers to SMD and FW

For validation of SSR markers, 52 germplasm lines representing the resistant and susceptible phenotypic classes based on the mean PDI were selected. The genomic DNA was extracted from two-week-old seedlings using the Cetyl-tri-methyl ammonium bromide (CTAB) method (Agbagwa *et al.,* 2012) and quantified on 0.8% gel using a known quantity of uncut λ DNA as a control. Further, the genomic DNA was diluted to 25-30 ng/µl for SSR marker analysis.

For the validation study, a total of 15 and 13 reported SSR markers linked to SMD and FW resistance were utilized, respectively. The PCR reaction mixture consisted of 1 µl of stock genomic DNA, 0.3 µl Taq DNA polymerase, 1.5 µl 10X TE buffer, 0.2 µl $MgCl_2$, 1.5 µl dNTPs and 0.5 µl of forward and reverse primer, making a total of 15 µl PCR mixture. Amplification was performed using the touchdown PCR method in a VeritiTM thermal cycler (Applied Biosystems). The amplified products were separated on a 2.5% agarose gel in 1X TBE buffer at 80V and visualized using the Bio-rad XR + gel documentation system. Based on amplicon size specific to resistant and susceptible checks, the amplified products were scored. Subsequently, single marker analysis using a t-test was conducted at each marker locus to compare the mean PDI of resistant and susceptible genotypes, aiming to identify significant differences between marker classes ($p<0.05$) (Darvasi *et al.,* 1993) and to validate the markers utilizing Microsoft Excel 2019.

RESULTS AND DISCUSSION

Assessment of genetic variability for different quantitative traits

The analysis of variance highlighted the presence of significant genetic differences among the genotypes for all traits. The mean sum of squares due to blocks were significant for all the traits except for test weight and pod-bearing length (Table 1 and Table 2). Within and across environment variability was visualized using Box-whisker plots. The length of the box and whisker lines represents the range, while the dot inside the box signifies the mean value of the trait (Fig 1). The descriptive statistics and genetic variability parameters for each environment are given in Table 3. The genotypes ICPL 19511 (48.91 g), ICPL 19514 (40.73 g) and ICPL 19493 (39.67 g) in EI while ICPL 19494 (46 g), ICPL 15057 (46 g) and ICPL 19511 (45.05 g) in EII and the genotypes ICPL 19511 (46.87 g), ICPL 15057 (44.39 g) and ICPL 19514 (42.09 g) across environments were found to be the high yielding genotypes that were consistent with the checks (BRG3 = 39.66 g and BRG5 = 42.38 g).

The estimated phenotypic coefficient of variation (PCV) was higher than the genotypic coefficient of variation (GCV) across all traits. Notably, high PCV values were recorded for traits such as number of primary branches $plant^{-1}$, number of secondary branches $plant^{-1}$, number of pods $plant^{-1}$, pod bearing length and seed yield $plant^{-1}$. Similarly, high GCV values were observed for the same traits ranging from 78.64% to 99.8% in EI and 78.54% to 99.72% in EII. Additionally, higher genetic advance as per cent of mean (GAM) was observed across all traits except days to 50% flowering (9.49 %) in EI. Higher GAM was observed for almost all the traits in EII except for days to 50 % flowering (9.48%) for which the lowest GAM was recorded and moderate GAM was observed for pod length (19.85%) and test weight (19.98).

Following Levene's test to account for homogeneity of variances, the 78 genotypes were then clustered together into nine distinct clusters using non-hierarchical *k* means clustering. Out of nine clusters, cluster III contained the maximum number of 17 genotypes and Cluster VIII had the minimum number of three genotypes. The distribution of genotypes in nine clusters is depicted in (Fig 2) and the distribution of genotypes in different clusters is represented in Table 4.

The analysis of variance between the clusters revealed the presence of significant variation among clusters Table 5. The estimated cluster means along with the range within each cluster for various traits are illustrated in Fig 3. The maximum inter-cluster distance was observed between cluster I and cluster VIII (7.03), followed by cluster I and cluster VII (6.88). The minimum inter-cluster distance was observed between cluster III and cluster VI (3.40) Table 6.

Screening for disease resistance

The mean per cent disease incidence for SMD on resistant (BRG3) and susceptible (ICP 8863) checks were 0 and 100%, respectively. Similarly, for FW, average disease incidence for resistant (BRG3) and susceptible (ICP 2376) checks were 0% and 79.0%, respectively. Upon disease reaction assessment (Fig 4), 17 genotypes were grouped as resistant (0-10.0% PDI) to SMD and seven genotypes for FW. Additionally, 64 (10.1-30% PDI for SMD) and 11 (10.1-20% for FW) genotypes displayed moderately resistant reaction, while 13 genotypes were moderately susceptible (20.1-40% PDI) to FW. Furthermore, 21 (30.1-100%) and 72 (40.1-100% PDI) genotypes were susceptible to SMD and FW, respectively. Six genotypes *viz*., ICPL 15023, ICPL 15063, ICPL 19480, ICPL 19482, ICPL 19489 and ICPL 19499 showed combined resistance, while seven genotypes ICPL 15014, ICPL 19472, ICPL 19477, ICPL 19487, ICPL 19495, ICPL 19540 and ICPL 19529 were moderately resistant to both SMD and FW. The frequency distribution was skewed for SMD (1.37) and FW (-0.07) as indicated by the significance of Shapiro Wilk's test for normality [SMD (p-value = 1.91×10^{-8}) and FW (p-value = 2.31×10^{-5})]. While the kurtosis value for SMD was 4.95 and for FW it was 1.78.

Validation of SSR markers

In this study, four SSR markers (AHSSR 50, AHSSR 34, AHSSR 20 and CcM 0588) out of 15 for SMD and seven markers (ASSR 23, ASSR 229, ASSR 363, CcGM 03681, HASSR 18, HASSR 121 and HASSR 128) out of 13 for FW exhibited polymorphism. These markers effectively distinguished the resistant and susceptible genotypes based

Table 1: Analysis of variance for yield and yield-related traits in the environment I (EI).

Source of variation	df	Mean sum of squares									
		DFF[a]	PH[b]	PB[c]	SB[d]	NPP[e]	NSP[f]	PBL[g]	PL[h]	TW[i]	YLD[j]
Block (ignoring treatment)	5	116.07**	854.3*	8.11**	17.46***	15823***	0.40**	572.3***	0.76*	1.56	49.89**
Treatment (eliminating block)	79	62.52**	624.2*	8.20**	15.75***	15804***	0.38**	306.7**	0.89**	2.66*	78.09***
Treatment adjusted check	1	52.08*	602.4*	3.00	7.12***	318*	0.24*	1252.6***	1.96**	7.30**	18.91*
Treatment adjusted test vs check	78	62.66***	624.5*	8.26**	15.86***	16003***	0.38**	294.5**	0.88**	2.60*	78.85***
Treatment (ignoring block)	79	69.6**	674.5*	8.65**	16.79***	16804***	0.41**	342.1**	0.92**	2.53*	81.0***
Block adjusted check	1	52.1*	602.4	3.00	7.12***	318*	0.24*	1252.6***	1.97**	7.30**	18.9*
Block adjusted treatment	77	36.8*	679.2*	7.29**	16.91***	15875***	0.24**	334.6**	0.49*	1.87*	54.2**
Block adjusted test vs check	1	2609.3***	389.4	119.4***	17.46***	104781***	12.98***	9.00	33.05***	48.58***	2206.3***
Block eliminating treatment	5	4.3	60	1	1.05*	31	0.037	12.8	0.38.	3.6*	4.3
Residuals	5	5.7	100	0.6	0.13	32	0.021	18.8	0.09	0.4	2.3
				SE							
Control Tr means	1.37	5.77	0.44	0.21	3.2	0.084	2.50	0.17	0.36	0.86	
Two test treatments same block	3.37	14.14	1.09	0.52	7.94	0.206	6.12	0.42	0.89	2.12	
Two test treatments different block	4.12	17.32	1.34	0.64	9.73	0.252	7.50	0.51	1.09	2.60	
Test vs check	3.15	15.38	1.02	0.49	7.43	0.193	5.73	0.39	0.83	1.98	
				CD							
Control Tr means	3.53	14.84	1.14	0.55	8.33	0.21	6.42	0.44	0.93	2.22	
Two test treatments same block	8.66	36.35	2.81	1.35	20.42	0.53	15.74	1.07	2.29	5.46	
Two test treatments different block	10.62	44.52	3.44	1.66	25.01	0.65	19.28	1.32	2.81	6.68	
Test vs check	8.11	16.31	2.63	1.26	19.10	0.49	14.73	1.008	2.15	5.10	
Coefficient of variance	2.167	5.48	8.54	5.90	3.23	3.90	7.64	5.98	5.60	5.26	

*** Significant at P= 0.001; ** Significant at P= 0.01; * Significant at P= 0.05.

[a]Days to 50% flowering, [b]Plant height (cm), [c]Number of primary branches plant^{-1}, [d]Number of secondary branches plant^{-1}, [e]Number of pods plant^{-1}, [f]Number of seeds pod^{-1}, [g]Pod bearing length (cm), [h]Pod length (cm), [i]Test weight (g) and [j]Seed yield plant^{-1} (g).

on amplicon size, as illustrated in Fig 5. To ascertain a significant association between the markers and disease response, a single-marker analysis was carried out using a t-test. The results revealed that only one marker (AHSSR 20) for SMD and four markers (ASSR 229, HASSR 121, HASSR 12, CcGM 03681) for FW effectively differentiated the germplasm lines based on p-value, as indicated in Table 7.

Yield comparison of genotypes with combined disease-resistance

The disease incidence of genotypes with combined resistance *viz*., ICPL 19499, ICPL 19489, ICPL 19482, ICPL 15023, ICPL 15063 and ICPL 19467 for SMD and FW along with the yield data are represented in Table 8. Among these genotypes, the performance of ICPL 15023 was comparable with the best check BRG3 based on the mean critical difference (5.37) across environments for grain yield.

Plant breeding success hinges largely on developing High-yielding varieties that possess resistance to pests and diseases. The analysis of variance of yield and yield-related traits reflected on the presence of high genetic variability among the genotypes based on the distribution of genotypes in two environments and across environments as depicted by box whisker plots in Fig 1. The traits days to 50% flowering, number of primary branches plant^{-1}, test weight and seed yield plant^{-1} displayed the symmetrical distribution while the traits plant height, number of secondary branches plant^{-1}, number of pods plant^{-1}, number of seeds pod^{-1}, pod bearing length and pod length displayed asymmetric distribution. A higher magnitude of selection response can be visualized for traits with symmetric distribution than the traits with skewed distribution (Katral *et al.,* 2022). The estimated genetic variability parameters indicated that PCV values were relatively higher than the GCV values, suggesting the significant influence of environmental factors on trait expression (Vanniarajan *et al.,* 2021; Parre and Raje, 2022). All the traits showed high heritability along with substantial GAM, except days to 50% flowering, implying predominance of additive gene action and facilitating effective selection for these traits with lesser environmental influence, as observed in the studies conducted by Pushpavalli *et al.* (2018) and Hemavathy *et al.* (2019).

The *k*-means clustering analysis grouped the genotypes, along with the checks, into nine clusters, revealing substantial diversity among them. The analysis of variance between these clusters further confirmed significant differences, highlighting distinctiveness among genotypes belonging to different clusters. Notably, clusters IX and V showed high divergence based on inter-cluster distance, while clusters X and III appeared less divergent and potentially related to each other. These findings align with those reported by (Kanavi *et al.*, 2020) in green gram, Amit *et al.* (2022) in chickpea and Nautiyal *et al.* (2025) in cowpea.

Screening for disease resistance

Most genotypes displayed moderate resistance to SMD and susceptibility to FW, with fewer resistant genotypes identified for both diseases. The distribution of genotypes was skewed for both SMD (positive) and FW (negative) diseases. The positive skewness indicates dominant and complementary gene action indicating that genetic gain could be increased by intensive selection of the genotypes at the tail end. While negative skewness indicates dominant and duplicate gene action indicating that the mild selection can be applied at the tail end of genotypes for rapid improvement of genotypes. Additionally, the distribution of SMD was leptokurtic, suggesting that resistance is controlled by a few genes, while the distribution of FW was platykurtic, indicating that resistance is influenced by many genes (Bassuony *et al.,* 2021). These findings differ from previous studies for SMD by Patil *et al.* (2016), Rajeswari *et al.* (2021) and Tharageshwari *et al.* (2019), which reported a higher number of genotypes in the susceptible class for SMD, albeit with a negatively skewed distribution. The negatively skewed distribution for FW was previously reported by Ashitha *et al.* (2016), Naik *et al.* (2017) and Nagaraja *et al.* (2016). Additionally, the root dip technique was found effective and efficient in identifying resistant genotypes based on reduced area and reduced time requirements (Ashitha *et al.,* 2016).

Validation of SSR markers

The validation of reported SSR markers linked to resistance to SMD and FW was carried out to suggest efficient markers to be used in marker-assisted selection programs. A total of four and seven markers were found polymorphic and differentiated the resistant and susceptible genotypes for SMD and FW disease, respectively. The AHSSR markers exhibited polymorphism for SMD resistance, as reported by Patil *et al.* (2016). The CcM markers identified by Gnanesh *et al.* (2011), were predominantly monomorphic in our study, except for CcM0588, possibly due to their distance from the QTLs or their specificity to certain genotypes (Behera *et al.,* 2020) or original populations (Geddam *et al.,* 2014). Hence, before their use, these CcM markers require validation in other populations. Among all markers, the AHSSR 20 (p-value = 0.00016) marker stands out as a significant marker with a p-value <0.05. Thus, can be used for selecting (SMD) resistant genotypes as indicated by single marker analysis.

Seven out of 13 markers were found to be polymorphic, consistent with the findings of Bohra *et al.* (2017) for CcGM 03681, Patil *et al.* (2017) for HASSR markers and Singh *et al.* (2013) and Singh *et al.* (2016) for ASSR markers. Among these markers, only four showed significant associations with FW resistance (HASSR121 with p-value <0.001, ASSR229 with p-value <0.01, HASSR128 and CcGM03681 with p-value<0.05) based on t-test results. The effectiveness of t test to carry out single marker analysis, for establishing marker trait association was proved effective in various other studies (Diwan *et al.,* 2022; Bhiza *et al.,* 2015). Hence, markers that were found to be significantly associated have the potential to be utilized in marker-assisted selection for the identification of resistant

Table 2: Analysis of variance for yield and yield-related traits in the environment II (EII).

Source of variation	df	Mean sum of squares									
		DFF[a]	PH[b]	PB[c]	SB[d]	NPP[e]	NSP[f]	PBL[g]	PL[h]	TW[i]	YLD[j]
Block (ignoring treatment)	5	94.36**	1072.2*	11.96**	23.99***	11771***	0.35**	142.60	0.71***	1.73	241.94***
Treatment (eliminating block)	79	65.2**	672.4*	8.63**	18.8***	11467***	0.30**	339.5*	0.51***	2.63*	72.78***
Treatment adjusted check	1	52.08*	602.4	3.00	7.12***	318*	0.24*	918.8**	0.75***	7.3**	25.76*
Treatment adjusted test vs check	78	65.37**	673.3*	8.7**	18.95***	11610***	0.30**	332.1*	0.51***	2.57*	73.38***
Treatment (ignoring block)	79	70.9**	736.5*	9.32**	20.26***	12210***	0.32**	348.57*	0.56***	2.51*	87.8***
Block adjusted check	1	52.08*	602.4	3.00	7.13***	318*	0.24*	918.75**	0.75***	7.30**	25.8*
Block adjusted treatment	77	37.01*	746.3*	8.71**	20.18***	11096***	0.21**	287*	0.26***	1.86*	68.3***
Block adjusted test vs check	1	2698.8***	116.7	62.93***	38.82***	109910***	8.98**	4528***	22.76***	47.66**	1648.8***
Block eliminating treatment	5	4.35	60	1	1.06*	31	0.037	0	0.012	3.6*	4.5
Residuals	5	5.68	100	0.6	0.14	32	0.021	40	0.004	0.4	2.8
				SE							
Control Tr means	1.37	5.77	0.44	0.21	3.24	0.08	3.65	0.036	0.36	0.95	
Two test treatments same block	3.37	14.14	1.09	0.52	7.94	0.20	8.94	0.089	0.89	2.35	
Two test treatments different block	4.12	17.32	1.34	0.64	9.73	0.25	10.95	0.11	1.09	2.87	
Test vs check	3.15	13.22	1.02	0.49	7.43	0.19	8.36	0.083	0.83	2.19	
				CD							
Control Tr means	3.53	14.84	1.14	0.55	8.33	0.21	9.38	0.09	0.93	2.46	
Two test treatments same block	8.66	36.35	2.81	1.35	20.42	0.53	22.99	0.22	2.29	6.04	
Two test treatments different block	10.61	44.52	3.44	1.66	25.02	0.65	28.15	0.28	2.81	7.0	
Test vs check	8.10	34.00	2.63	1.26	19.10	0.49	21.50	0.21	2.15	5.65	
Coefficient of variance	2.16	5.24	7.84	5.43	3.27	3.74	8.80	1.15	5.59	5.55	

*** Significant at P= 0.001; ** Significant at P= 0.01; * Significant at P= 0.05.

[a]Days to 50% flowering, [b]Plant height (cm), [c]Number of primary branches plant^{-1}, [d]Number of secondary branches plant^{-1}, [e]Number of pods plant^{-1}, [f]Number of seeds pod^{-1}, [g]Pod bearing length (cm), [h]Pod length (cm), [i]Test weight (g) and [j]Seed yield plant^{-1} (g).

Table 3: Descriptive statistics and genetic variability parameters in environment I (EI) and environment II (EII).

Characters	Mean ± SE_m		Range				PCV (%)[c]		GCV (%)[d]		H (%)[e]		GAM (%)[f]	
	EI	EII	EI		EII		EI	EII	EI	EII	EI	EII	EI	EII
			Min[a]	Max[b]	Min[a]	Max[b]								
Days to 50% flowering	111.69±0.74	111.95±0.74	94.17	129.75	94.16	131.75	5.43	5.43	4.99	4.99	84.57	84.64	9.49	9.48
Plant height	181.64±2.85	190.88±3.00	73.67	220.00	87.00	224.00	14.34	14.31	13.32	13.31	85.27	86.60	25.24	25.56
Number of primary branches $plant^{-1}$	8.7±0.31	9.6±0.32	3.00	17.33	4.00	17.17	29.73	30.73	29.65	29.63	91.76	93.11	58.76	59.03
Number of secondary branches $plant^{-1}$	6.46±0.47	7.08±0.51	0.42	18.39	0.40	19.75	63.67	63.46	63.41	63.24	99.17	99.31	130.28	130.02
Number of pods $plant^{-1}$	162.65±14.01	160.28±11.73	23.16	584.66	13.66	490.00	77.47	65.72	77.39	65.62	99.8	99.72	159.50	135.19
Number of seeds pod^{-1}	3.62±0.06	3.8±0.055	1.03	5.03	2.03	5.03	13.78	12.27	13.17	11.65	91.42	90.19	25.99	22.83
Pod bearing length	56.79±2.02	74.09±1.91	6.71	95.80	30.00	112.00	32.21	22.86	31.29	21.21	94.39	86.06	62.72	40.58
Pod length	4.76±0.11	5.31±0.06	0.97	6.90	3.95	7.00	14.66	9.77	13.27	9.70	81.95	98.51	24.79	19.85
Test weight	11.05±0.22	11.07±0.24	6.90	15.20	7.10	15.10	12.38	12.33	10.98	10.93	78.64	78.54	20.09	19.98
Seed yield $plant^{-1}$	26.97±0.88	28.57±0.99	11.69	48.45	12.18	48.23	27.28	28.92	26.71	28.33	95.83	95.95	53.95	57.25

[a]Minimum value, [b]maximum value, [c]Phenotypic coefficient of variation, [d]Genotypic coefficient of variation, [e]Broad sense heritability, [f]Genetic advance as per cent of mean.

Table 4: Distribution of genotypes in nine clusters obtained using *k* means clustering.

Clusters	Number of genotypes	Genotypes
I	4	ICPL 19516, ICPL 19527, ICPL 19495 and ICPL 19527
II	13	ICPL 15079, 19512, ICPL 19535, ICPL 19470, ICPL 19481, ICPL 19496, ICPL 15006, ICPL 19510, ICPL 15023, ICP8863, ICPL 19502, ICPL 19505 and ICPL87119
III	17	ICPL 19517, ICPL 19525, ICPL 19536, ICPL 19539, ICPL 19474, ICPL 19476, ICPL 19482, ICPL 19485, ICPL 19487, ICPL 19486, ICPL 19488, ICPL 19490, ICPL 19500, ICPL 19499, ICPL 15003 and ICPL 19508
IV	8	ICPL 19515, ICPL 19530, ICPL 19541, ICPL 19475, ICPL 19483, ICPL 15063 and ICPL 15067
V	8	ICPL 19518, ICPL 19537, ICPL 19547, ICPL 19493, ICPL 15010, ICPL 15058, ICPL 15062 and ICPL 19503
VI	15	ICPL 19519, ICPL 19520, ICPL 19526, ICPL 19533, ICPL 19544, ICPL 19468, ICPL 19469, ICPL 19472, ICPL 19478, ICPL 19477, ICPL 19492, ICPL 19497, ICPL 19498, ICPL 15007 and ICPL 19504
VII	6	ICPL 19489, ICPL 19467, ICPL 19545, ICPL 15060, ICPL 19506 and ICPL 19509
VIII	3	ICPL 19494, BRG3 and BRG5
IX	6	ICPL 19511, ICPL 19514, ICPL 19524, ICPL 19542, ICPL 15014 and ICPL 15057

Table 5: Analysis of variance for yield and yield-attributing traits between the clusters.

Source of variation	DFF[a]	PH[b]	PB[c]	SB[d]	PP[e]	SP[f]	PBL[g]	PL[h]	TW[i]	YLD[j]
Between clusters	205.12***	4512***	20.11***	135.54***	85942***	0.95***	1414.89***	1.66***	20.12***	376.88***
Within clusters	23.74	234.60	4.28	5.51	4276	0.10	78.07	0.18	2.18	28.82

***Significant at P= 0.001; **Significant at P= 0.01; *Significant at P= 0.05.

[a]Days to 50% flowering, [b]Plant height (cm), [c]Number of primary branches plant^{-1}, [d]Number of secondary branches plant^{-1}, [e]Number of pods plant^{-1}, [f]Number of seeds pod^{-1}, [g]Pod bearing length (cm), [h]Pod length (cm), [i]Test weight (g) and [j]Seed yield plant^{-1} (g).

Table 6: Intra and inter-cluster distance among the nine clusters.

Clusters	C I	C II	C III	CIV	CV	C VI	C VII	C VIII	C IX
CI	0.00								
CII	5.50	0.00							
CIII	5.19	4.02	0.00						
CIV	5.16	4.23	3.88	0.00					
CV	6.03	3.53	3.88	4.39	0.00				
CVI	5.68	3.44	3.40	4.40	3.83	0.00			
CVII	6.88	4.56	5.04	6.08	4.57	4.91	0.00		
CVIII	7.03	4.31	6.15	6.66	5.05	4.95	5.48	0.00	
CIX	6.63	4.16	5.33	5.44	4.34	5.65	5.07	5.74	0.00

C = Cluster.

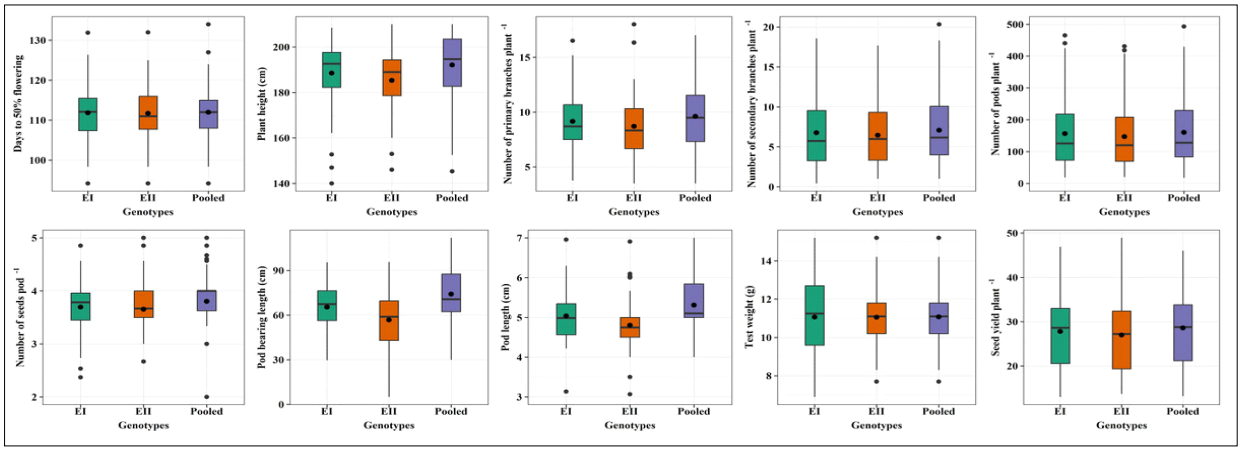


Fig 1: Box-whisker plots depicting variability in each environment and across environments for yield and yield-attributing traits in pigeonpea.

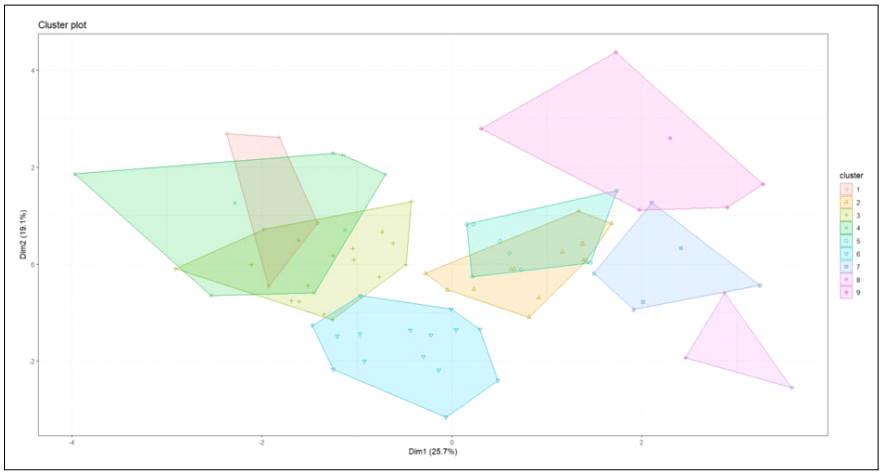


Fig 2: *k* means clustering of pigeonpea genotypes based on yield and yield-attributing traits.

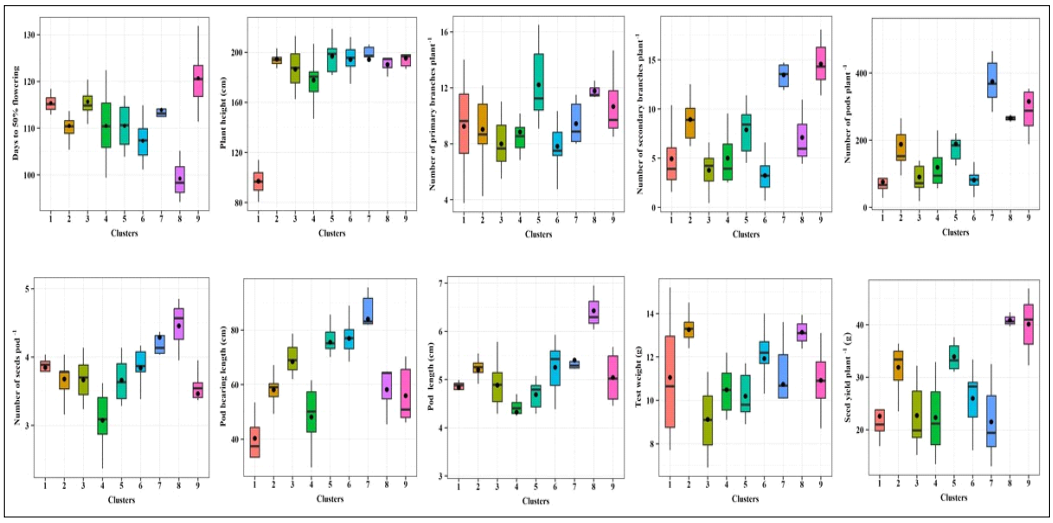


Fig 3: Box-whisker plots showing the variation of genotypes within each cluster for yield and yield-attributing traits.

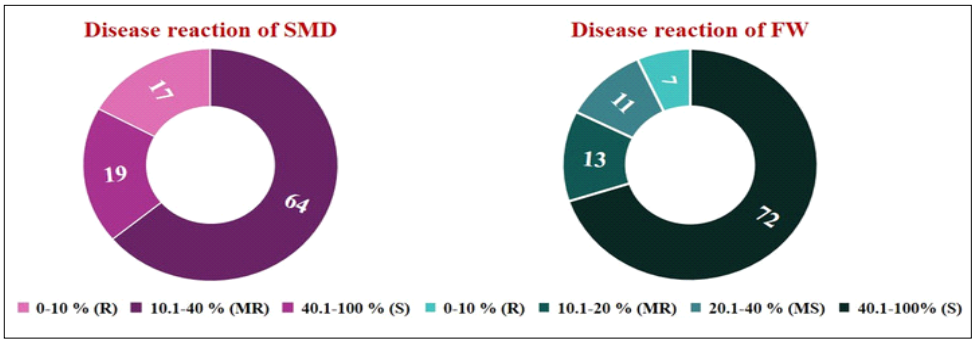
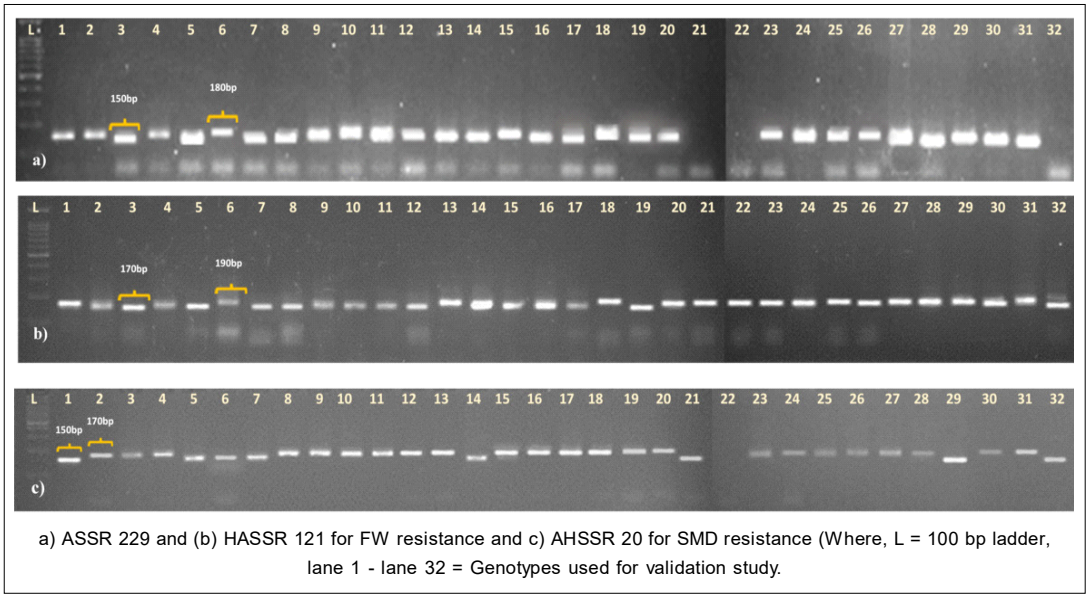


Fig 4: Distribution of genotypes into different disease classes based on their response to SMD and FW.



a) ASSR 229 and (b) HASSR 121 for FW resistance and c) AHSSR 20 for SMD resistance (Where, L = 100 bp ladder, lane 1 - lane 32 = Genotypes used for validation study.

Fig 5: Gel pictures depicting the amplification of significantly associated markers.

Table 7: Association between the SSR markers and resistance to SMD and FW disease.

Markers	p-value
SMD	
CcM0588	0.5671
AHSSR 50	0.2587
AHSSR 34	0.6833
AHSSR 20	0.00016***
FW	
ASSR 23	0.4919
ASSR 229	0.0025**
ASSR 363	0.9637
HASSR 18	0.2772
HASSR 121	0.0009***
HASSR 128	0.0201*
CcGM03681	0.0388*

***Significant at P= 0.001; **Significant at P= 0.01; *Significant at P= 0.05.

Table 8: Performance of combined disease-resistant genotypes for yield and yield-related traits.

Genotypes	Seed yield plant^{-1} (g)	PDI	
		SMD	FW
ICPL 15023	34.92	10	0
ICPL 15063	17.22	0	0
ICPL 19467	21.15	0	10
ICPL 19482	26.88	0	10
ICPL 19489	16.50	10	0
ICPL 19499	18.54	10	10
BRG3 (Ch)	39.66	0	0

genotypes, thereby serving as valuable tools in the quest to identify potential sources of resistance.

CONCLUSION

Among ten yield attributing traits studied in the newly developed germplasm lines of pigeonpea, high PCV, GCV, heritability and genetic advance as per cent mean was recorded by the traits number of primary branches plant^{-1}, number of secondary branches plant^{-1}, number of pods plant^{-1}, pod bearing length and seed yield plant^{-1}. Results from *k*-means clustering indicated the presence of a higher magnitude of genetic diversity. Six germplasm lines displayed combined resistance [ICPL 19499, ICPL 19489, ICPL 19482, ICPL 15023, ICPL 15063 and ICPL 19467] against SMD and FW. The line, ICPL 15023 showed resistance to both diseases and high-yielding. The markers, AHSSR 20 for SMD and ASSR 229, HASSR 121, HASSR 128 and CcGM 03681 for FW exhibited significant associations with disease response, indicating their potential for identifying resistant sources. The genotypes *viz*., ICPL 19511 and ICPL 15057 for yield and ICPL 15023, ICPL 15063, ICPL 19499, ICPL 19482, ICPL 19489 and ICPL 19467 with combined resistance and high grain yield could be considered for release after extensive yield trials and can also be used in breeding programs for disease resistance introgression.

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Authors' contribution

Conceptualization of research (HC Lohithaswa); Designing of the experiments (HC Lohithaswa, Prakash Gangashetty, HB Shruthi); Execution of field/lab experiments and data collection (GS Sinchana Kashyap, HC Lohithaswa, Santoshkumari Banakara, MS Sowmya); Analysis of data and interpretation (GS Sinchana Kashyap, HC Lohithaswa, MG Mallikarjuna); Preparation of the manuscript (GS Sinchana Kashyap, Santoshkumari Banakara, MS Sowmya, HC Lohithaswa, MG Mallikarjuna, Prakash Gangashetty, HB Shruthi).

Conflict of interest

The authors have declared that no competing or conflict of interest exists.

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