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Genetic Diversity Analysis in Extra Early Pigeonpea [*Cajanus cajan* (L.)] Genotypes using SSR Markers

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ABSTRACT

Background: The present study aims at assessment of genetic diversity in 43 extra early pigeonpea genotypes with 41 simple sequence repeat markers.

Methods: In the present investigation, 41 HASSR markers were used to assess the genetic diversity. Similarity matrices were utilized to construct the unweighted pair group method with Arithmetic average dendrogram using NTSYs PC software.

Result: Out of 41 HASSR markers, 21 markers were showed distinct banding pattern, while 11 markers were showed monomorphic banding pattern and remaining 9 markers showed no banding pattern or not amplified. Cluster analysis done using HASSR markers revealed that ICPL87119 was found to be the most diverse. This genotype also found to be resistant to wilt. Based on similarity coefficients and cluster analysis genotypes ICPL87119, ICPL84031, ICPL88014, ICPL161, ICPL87091 and CORG9701 were genetically more distant from other genotypes studied and these varieties can be used for their desirable characteristics in breeding programs for pigeonpea improvement.

Key words: Correlation, Genetic diversity, Pigeonpea, SSR markers.

INTRODUCTION

Pigeonpea is an often cross-pollinated diploid (2n=2X=22) crop with 833.07 Mb genome size (Varshney *et al.* 2012a). Pigeonpea plays an important role in sustainable agriculture, because of its multiple usages in food, fodder, soil conservation, crop-livestock integrated systems, reclaiming of degraded pastures, symbiotic nitrogen fixation and has an important role in vegetarian diet in developing countries by ensuring high supply of vitamin B carotene, ascorbic acid and rich protein (22%) (Varshney *et al.* 2013). Desirable level of productivity in pigeonpea can be exploited by selecting the existing variability among indigenous germplasm of pigeonpea. However, to attain further breakthrough in increasing yield and improving stability in future cultivars, new variability needs to be tapped and incorporated into cultivars. Using crop wild relatives in breeding programme is a long and laborious process that is typically much more difficult than breeding with cultivated crop varieties. Many plant breeders avoid the use of crop wild relatives for this reason. The first step towards using crop wild relatives in breeding is prebreeding, an essential component for this purpose 43 extra early genotypes are utilized in this research work.

Assessment of genetic diversity has traditionally been made through morphological characters that are often limited in number, have complex inheritance and vulnerable to environmental conditions. DNA marker overcome this ill effect as they are abundant in nature, stable and not influenced by environmental fluctuations. Among the DNA markers, simple sequence repeat (SSR) or microsatellite marker is one of the most useful genetic marker systems that use PCR technique to identify differences in microsatellite repeat units. SSR markers are widely used because of its co-dominant, multi allelic, high polymorphism, reproducibility, abundant informativeness, convenience of assay by PCR and distribution throughout the genome, independent of environments, independent of tissue effects and providing more precise characterization of genotypes and measurement of genetic relationships than other markers (Gupta and Varshney, 2000; Hari *et al.* 2017). SSRs with ≥20 nucleotides and <20 nucleotides are referred to as Class I (hypervariable) and Class II, respectively. The significance of hyper-variable SSRs in pigeonpea has been established owing to their ease of scoring in simple agarose gel (Bohra *et al.* 2017). Keeping these points in view, the present investigation was carried out with an objective of understanding the genetic diversity among 43 pigeonpea accessions using hyper variable SSR markers.

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MATERIALS AND METHODS

Collection of plant material

A set of 43 pigeonpea genotypes were obtained from ICRISAT, Patancheru and these genotypes were raised during 2016-17 following standard Agronomic Practices at Regional Agricultural Research Station, Waragal, PJTSAU, Telangana. Leaf samples were collected from all the genotypes at 30 Days after sowing (DAS) for DNA isolation (Table 1).

DNA isolation and PCR analysis

Genomic DNA from leaf samples was isolated by following the standard protocol as per the procedure described by Murray and Thompson (1986), with few modifications. Final concentration of 30 ng/µl of genomic DNA was used for PCR

Table 1: List of pigeonpea genotypes and their characteristics.

Variety	Plant growth habit	Leaf shape	Flower colour	Days to 50% flower	Seed colour
ICPL-87119	Indeterminate	Ablong	Yellow	120 Days	Red
UPAS-120	Indeterminate	Ablong	Yellow	91 Days	Red
ICPL 11263	Determinate	Ablong	Yellow	56 Days	Red
ICPL11255	Determinate	Ablong	Yellow	56 Days	Red
ICPL11253	Determinate	Ablong	Yellow	58 Days	Red
ICPL11256	Determinate	Ablong	Yellow	58 Days	Red
ICPL11259	Determinate	Ablong	Yellow	56 Days	Red
ICPL20338	Determinate	Ablong	Yellow	58 Days	Red
ICPL20340	Determinate	Ablong	Yellow	61 Days	Red
ICPL11251	Determinate	Ablong	Yellow	62 Days	Red
ICPL11258	Determinate	Ablong	Yellow	68 Days	Red
ICPL11260	Determinate	Ablong	Yellow	58 Days	Red
ICPL11265	Determinate	Narrowly ablong	Yellow	58 Days	Red
ICPL11254	Determinate	Ablong	Light yellow	58 Days	Red
ICPL11274	Determinate	Ablong	Yellow	59 Days	Red
ICPL11276	Indeterminate	Ablong	Yellow	59 Days	Red
ICPL11298	Indeterminate	Ablong	Yellow	59 Days	Red
ICPL11318	Indeterminate	Ablong	Yellow	70 Days	Red
ICPL11300	Indeterminate	Narrow	Yellow	70 Days	Red
ICPL11326	Indeterminate	Narrowly ablong	Yellow	76 Days	Red
ICPL11279	Indeterminate	ablong	Yellow	76 Days	Red
ICPL11285	Indeterminate	Ablong	Yellow	73 Days	Red
ICPL11242	Indeterminate	Narrow	Yellow	71 Days	Red
ICPL11292	Indeterminate	Ablong	Yellow	77 Days	Red
ICPL11245	Indeterminate	Narrow	Yellow	63 Days	Red
ICPL20333	Indeterminate	Narrowly ablong	Yellow	63 Days	Red
ICPL11324	Indeterminate	Ablong	Yellow	63 Days	Red
ICPL20335	Indeterminate	Narrow	Yellow	71 Days	Red
ICPL20329	Indeterminate	Ablong	Yellow	70 Days	Red
ICPL20325	Indeterminate	Ablong	Yellow	71 Days	Red
ICPL20328	Indeterminate	Ablong	Yellow	67 Days	Red
ICPL11313	Indeterminate	Narrowly ablong	Yellow	75 Days	Red
ICPL11333	Indeterminate	Narrow	Yellow	75 Days	Red
ICPL11299	Indeterminate	Narrow	Yellow	67 Days	Red
ICPL11306	Indeterminate	Ablong	Yellow	64 Days	Red
ICPL11303	Indeterminate	Ablong	Yellow	75 Days	Red
ICPL87	Determinate	Ablong	Yellow	75 Days	Red
ICPL88039	Indeterminate	Narrowly ablong	Yellow	100 Days	Red
ICPL84031	Determinate	Ablong	Yellow	106 Days	Red
ICPL88034	Indeterminate	Narrow	Yellow	98 Days	Red
ICPL161	Indeterminate	Narrow	Yellow	106 Days	Red
ICPL87091	Determinate	Ablong	Red	100 Days	White
CORG-9701	Indeterminate	Ablong	Yellow	92 Days	Red

(Eppendorf) amplification. PCR was performed using 1 U of Taq DNA polymerase (Fermentas, Lithuania) and 1x PCR buffer (Genei, India) in 10-µl reaction volume with a thermal profile of 94°C for 5 min (initial denaturation), followed by 35 cycles of denaturation at 94°C for 1 minute, annealing temperature (Table 2) for 1 min, extension at 72°C for 2 min and a final extension of 7 min at 72°C. The amplified products were electrophoretically resolved on 4% Seakem LE® Agarose (Lonza, USA), containing 0.5 mg/ml of ethidium bromide in 0.5x TBE buffer and visualized under UV.

Data analysis

Allele number was given and scored according to its presence or absence, based on difference in molecular weight. Only the clear and unambiguous bands were scored. 41 markers were scored for the presence (1) and absence (0) of the corresponding band among the genotypes. Consequently, a data matrix comprising '1' and '0' was formed and subjected to further analysis. Further processing of data was done by carrying out sequential agglomerative hierarchical non-overlapping clustering (SAHN), on squared Euclidean distance matrix. Similarity matrix was done using Jaccard's coefficient, in which similarity matrices were utilized to construct the UPGMA (Unweighted Pair Group Method with Arithmetic average) dendrogram. Data analysis was done using NTSYs PC (Rohlf, 1998).

RESULTS AND DISCUSSION

The present investigation envisaged the degree of genetic diversity based on marker data in forty three genotypes of pigeonpea (Fig 1). Genetic diversity/ relatedness among the genotypes was assessed on the basis of Polymorphic information content (PIC) value. Out of 41 HASSR markers (Table 2), 21 markers (Table 3) were completely amplified and a total of 193 alleles were found. In the remaining markers, some were amplified and some were not amplified. Polymorphic information content (PIC Value) of SSR markers was calculated (Table 2). It ranged from 0.87 (HASSR 116) to 0.60 (HASSR-22, HASSR-29 and HASSR-68) with an average of 0.74. Molecular polymorphism was 58.8% with 21 HASSR primers indicating the low level of genetic variation among the varieties (Table 3). The polymorphic bands were scored visually as present (1) or absent (0) on a binary matrix. Genetic similarity between the varieties was estimated using Jaccards Coefficient of similarity index. Dendrogram was performed using the Unweighted Pair Group Method with an Arithmetic mean (UPGMA) algorithm and the NTSYS software (Fig 2).

Cluster analysis

Cluster analysis of the genotypes is depicted in Fig 2. The genotypes were grouped into two main clusters *i.e.* cluster

Fig 1A: SSR Amplification of 43 pigeonpea varieties with HASSR 236.

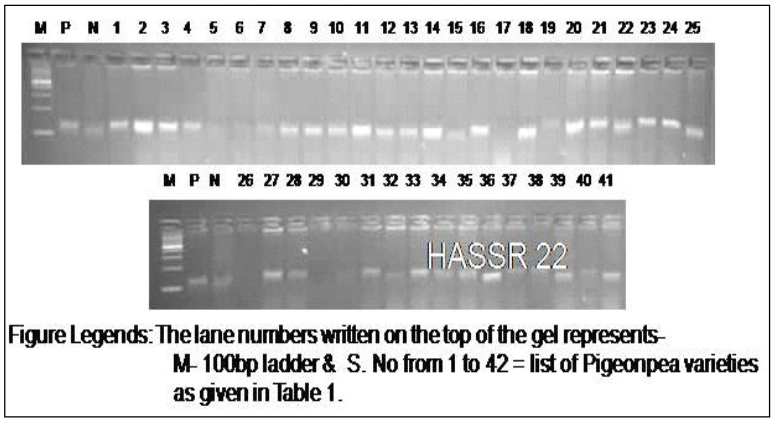

Fig 1B: SSR amplification of 43 pigeonpea varieties with HASSR 22.

Table 2: Details of HASSR markers used for diversity analysis among 43 genotypes of pigeonpea.

Primer name	Sequence of the primers (5'-3')	Annealing temperature (°C)	PCR amplicon size (bp)
HASSR1 F	CCTGTGACTCAACTCAATCTC	61	170-190
HASSR1 R	TGGAATACCGAATTAATGCTC		
HASSR5 F	GATAAGACCTTTTCACAAGCA	57	100-130
HASSR5 R	GATAAGACCTTTTCACAAGCA		
HASSR10 F	TTTTAACATCAAGGTTCCGTA	58	150-180
HASSR10 R	AATTCTTCTAAACATGCACCT		
HASSR11 F	ATTTTGGAGGATACAGCATTT	56	150
HASSR11 R	ACCATCACGAGTATATCTCCA		
HASSR21 F	GCAGGTCAGTTGCTTAACATA	59	180-200
HASSR21 R	AAGGCCAGAAATAACTTCTTC		
HASSR22 F	TCACAAACACAACACAACAAT	59	120-160
HASSR22 R	CTCTCTGCAGTTCTGGAATAC		
HASSR23 F	AAAGCTATGGAGCAATAGAAGA	56	160-195
HASSR23 R	TAATGGGTTGACCAGAAATTA		
HASSR29 F	AGAACACACAAAATGTAAAAGG	60	110-190
HASSR29 R	CTAGTGTATGGCATCACCATC		
HASSR37 F	TCAAAACTCCTCAGGTAATAAAA	59	100-160
HASSR37 R	ACTGCATTTAGTTTTGGACAG		
HASSR45 F	GGTGGAAGGAATCTTTAACTT	57	160-190
HASSR45 R	ACTTTCCTTTGATGCTTTTTC		
HASSR52 F	TCCCTTTTGAATAAAGGAGAC	58	140-180
HASSR52 R	TCCACACAACAAGGTTTAGAT		
HASSR68 F	ATCTTTTGGGTTGGTAAATTC	56	170-200
HASSR68 R	CGTTCTTCATCTTTTCACAAT		
HASSR71 F	TGTTGAAGCGAAAATAACAA	55	160-190
HASSR71 R	ACCGTATATTGTCCTCTGGAT		
HASSR86 F	GGTATTGCCTCCAAATACAAT	57	120-160
HASSR86 R	AAACTTCGATCAAAAGGAGAT		
HASSR90 F	GGGTTTATAACTTGGAAATGC	58	170-190
HASSR90 R	ACTTTTGTCCCATCTGTTTTC		
HASSR91 F	GTGACCTTTCGAGAGAAAAAT	60	110-150
HASSR91 R	TTAGTGGAGTGAGAGTCATGG		
HASSR92 F	GTGACCTTTCGAGAGAAAAAT	60	150-200
HASSR92 R	TTAGTGGAGTGAGAGTCATGG		
HASSR110 F	GTGAATTTTAGTACACAAGACAA	58	180
HASSR110 R	AAGTGATCGAATAATGGTGTG		
HASSR114 F	TACTGGTGATGATTGTGACAG	58	120-190
HASSR114 R	AATTCAACTCTAACATCTTCTGA		
HASSR116 F	TGAGATCTGGTCAAGGGTTA	58	180-190
HASSR116 R	GGTTGCATCTTCTTTTCTCTT		
HASSR143 F	ATTGACTCAACCTCCCTTTAG	58	160-210
HASSR143R	TTGAAGTAAATGGATCATTCC		
HASSR155 F	ACTTGTTGTTGACCCGACT	58	150-170
HASSR155 R	CTAAATAAGAGTAACAATGAGCA		
HASSR160 F	TGAATCACGTCTTAGATTTTAAC	58	115-160
HASSR160 R	TGTATTCCCAACATTATTTCG		
HASSR181 F	ATGACCCAACTTGACTAGGTT	58	120-180
HASSR181 R	ATGACCCAACTTGACTAGGTT		
HASSR189 F	CTTAGTGGAAGGAATTGGAGT	58	200
HASSR189 R	TGGCATAACAGTACGGATAGTA		

Table 2: Continue.....

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HASSR194 F	CGTGCCTTGCTATTATTTTTA	57	140-200
HASSR194 R	TGGTGTCCATATTTTTCAGAC		
HASSR219 F	GATGTGGATGTGGATGTGT	57	105-160
HASSR219 R	ACTTGATTATCCCCTTCCTC		
HASSR223 F	CACTTGACACTTCCAAAAGTT	56	140-160
HASSR223 R	TTCAATACCAGGCTGCTG		
HASSR224 F	CTAAGCCCATACCCCATAGT	60	160-180
HASSR224 R	ATACCACTTCCTTTCTCGAAC		
HASSR229 F	CAAGATAAATCCAACCAATGA	57	160-210
HASSR229 R	TTTTTCCTATTAGCTGAGTCG		
HASSR230 F	GGGTAGCATTAGAGGGTTTTA	60	130-160
HASSR230 R	AACCTATCCCATCTCTCTTGA		
HASSR231 F	TGAGCAAATAATCATTCAAGC	59	100-150
HASSR231 R	CCTGTTACTGACCTGACTCAA		
HASSR236 F	AGAGAGGGAAGAAATAAATGC	58	150-180
HASSR236 R	AAGAAGCGAGATCTACAACAA		
HASSR267 F	TATATCACCGTGTAATGCACA	58	145-195
HASSR267 R	TTATAGAATATGTCGCGAATTG		
HASSR271 F	GACAGATTGATTTCATTGGTT	59	160-190
HASSR271 R	CTCTAACAATAACGATGATGATG		
HASSR282 F	AACGAATTTTATCTCTCTCACAC	61	105-155
HASSR282 R	AGGTATTTCTTGGATGTTATCG		
HASSR289 F	ACAACATTGCATACCGTAATC	57	150-180
HASSR289 R	TGGTGGATTTTTGTGTTAGAT		
HASSR 294 F	CATTTCCTGATTAAACACGTC	57	150
HASSR 294 R	AAGGAGGTTTGGAGTATGAAG		
HASSR303 F	AGCAGCTTTTGATTCACAATA	57	180-200
HASSR303 R	CCTGCAGATAAAGTCGCTAT		
HASSR 305 F	GTAGGAAGTGTGGTGAAAATG	58	160-200
HASSR 305 R	TTTTACTGCTCCAACATTAGC		
HASSR 323 F	TGGAAATTCAGAGTAATATGTTTCC	62	230-260
HASSR 323 R	GGTCATGTTTGGTGGTCAGA		

A cluster and cluster B. Cluster A consists of only one genotype *viz*., ICPL 87119 and it showed 28% of similarity with Cluster B. Cluster B is further divided into sub clusters *i.e*. sub cluster B_1 and B_2 at 30% of similarity. The sub cluster B_1 is further divided into sub cluster $B_{1.1}$ and sub cluster $B_{1.2}$ at 35% of similarity. The sub cluster $B_{1.1}$ had 5 genotypes *viz*., UPAS 190, ICPL 11155, ICPL11161, ICPL11151 and ICPL11156. The sub cluster $B_{1.2}$ had 32 genotypes *viz*., ICPL11159, ICPL10140, ICPL11100, ICPL1116, ICPL10116, ICPL11151, ICPL11158, ICPL11160, ICPL11111, ICPL11199, ICPL11106, ICPL 11174, ICPL11198, ICPL11176, ICPL11118, ICPL10118, ICPL11111, ICPL11191, ICPL 10115, ICPL11114, ICPL10111, ICPL10119, ICPL10115, ICPL11165, ICPL11154, ICPL11185, ICPL11101, ICPL87, ICPL88019 and ICPL11141, while, sub cluster B2 had 5 genotypes *viz*., ICPL84031, ICPL88034, ICPL161, ICPL87091 and CORG9701. In sub cluster B2, the pigeonpea genotypes ICPL 84031and ICPL 88014 showed 68% of similarity, while the pigeonpea genotypes ICPL 87091 and CORG 9701were also showed 68% of similarity and in combination showed 50% of similarity. In Dendrogram the pigeonpea genotypes present in sub cluster B1.1 *viz*., ICPL 10118 and ICPL 11111 were showed highest percent (91%) of similarity.

It is observed from the study that the genotype ICPL 87119 was found to be the most diverse as it occupied a single cluster. It is also interesting to observe that it is resistant to wilt. Hence based on cluster analysis, the identified diverse pigeonpea genotypes can be effectively selected for carrying out various breeding and crop improvement programmes. The study clearly indicated that SSR marker profiles were best-suitable for assessing genetic relationships among pigeonpea genotypes. Based on similarity coefficients and cluster analysis pigeonpea genotypes ICPL 84031, ICPL88034, ICPL161, ICPL87091 and CORG9701 were genetically more distant from other pigeonpea genotypes and these varieties can be used for their desirable characteristics in breeding programs for Pigeonpea improvement (Fig 2).

Earlier, assessment of the genetic variation in pigeonpea has been carried out using different types of molecular markers including random amplified polymorphic DNA (RAPD) (Sarika Shende and Anand Raut; 2013), Resistance gene analog (RGA)-anchored amplified fragment length polymorphism (AFLP-RGA) (Patil *et al.* 2014) and simple sequence repeat (SSR) (Bohra *et al.* 2017, Suman *et al.* 2019 and Pankaj Sharma *et al.* 2020). Similar to our results, the narrow genetic base of the domesticated pigeonpea was also evident from analyses based on other DNA marker systems such as RAPD (Ratnaparkhe *et al.*1995), RFLP (Nadimpalli *et al.* 1993), AFLP (Panguluri *et al.*2006), DArT (Yang *et al.* 2006), ISR (Kudapa *et al.* 2012) and SNP (Kassa *et al.* 2012). As compared to the earlier successful reports, the number of polymorphic and informative markers used for genetic diversity analysis in the present investigation is more.

Table 3: List of 21 polymorphic markers with their sequence, annealing temperature and PIC.

Primer name	Sequence of the primers (5'- 3')	Annealing temperature	PCR amplicon size (bp)	PIC (Polymorphic information content)
HASSR 1 F	CCTGTGACTCAACTCAATCTC	61	170-190	0.64
HASSR 1 R	TGGAATACCGAATTAATGCTC			
HASSR 10 F	TTTTAACATCAAGGTTCCGTA	58	150-180	0.75
HASSR 10 R	AATTCTTCTAAACATGCACCT			
HASSR 21 F	GCAGGTCAGTTGCTTAACATA	59	180-200	0.75
HASSR 21 R	AAGGCCAGAAATAACTTCTTC			
HASSR 22 F	TCACAAACACAACACAACAAT	59	120-160	0.60
HASSR 22 R	CTCTCTGCAGTTCTGGAATAC			
HASSR 29 F	AGAACACACAAAATGTAAAAGG	60	110-190	0.60
HASSR 29 R	CTAGTGTATGGCATCACCATC			
HASSR 45 F	GGTGGAAGGAATCTTTAACTT	57	160-190	0.70
HASSR 45 R	ACTTTCCTTTGATGCTTTTTC			
HASSR 52 F	GCGGATCGTTACACTTTTA	58	200	0.86
HASSR 52 R	AGACGTTGCTTAAGTGTCTCA			
HASSR 68 F	ATCTTTTGGGTTGGTAAATTC	56	170-200	0.60
HASSR 68 R	CGTTCTTCATCTTTTCACAAT			
HASSR 86 F	GGTATTGCCTCCAAATACAAT	57	120-160	0.74
HASSR 86 R	AAACTTCGATCAAAAGGAGAT			
HASSR 110 F	GTGAATTTTAGTACACAAGACAA	58	180	0.74
HASSR 110 R	AAGTGATCGAATAATGGTGTG			
HASSR 114 F	TACTGGTGATGATTGTGACAG	58	120-190	0.75
HASSR 114 R	AATTCAACTCTAACATCTTCTGA			
HASSR 116 F	TGAGATCTGGTCAAGGGTTA	58	180-190	0.87
HASSR 116 R	GGTTGCATCTTCTTTTCTCTT			
HASSR 155 F	ACTTGTTGTTGACCCGACT	58	150-170	0.84
HASSR 155 R	CTAAATAAGAGTAACAATGAGCA			
HASSR 181 F	ATGACCCAACTTGACTAGGTT	58	120-180	0.85
HASSR 181 R	ATGACCCAACTTGACTAGGTT			
HASSR 189 F	CTTAGTGGAAGGAATTGGAGT	58	200	0.75
HASSR 189 R	TGGCATAACAGTACGGATAGTA			
HASSR 224 F	CTAAGCCCATACCCCATAGT	60	160-180	0.75
HASSR 224 R	ATACCACTTCCTTTCTCGAAC			
HASSR 230 F	GGGTAGCATTAGAGGGTTTTA	60	130-160	0.70
HASSR 230 R	AACCTATCCCATCTCTCTTGA			
HASSR 236 F	AGAGAGGGAAGAAATAAATGC	58	150-180	0.85
HASSR 236 R	AAGAAGCGAGATCTACAACAA			
HASSR 289 F	ACAACATTGCATACCGTAATC	57	150-180	0.72
HASSR 289 R	TGGTGGATTTTTGTGTTAGAT			
HASSR 294 F	CATTTCCTGATTAAACACGTC	57	150	0.85
HASSR 294 R	AAGGAGGTTTGGAGTATGAAG			
HASSR 303 F	AGCAGCTTTTGATTCACAATA	57	180-200	0.70
HASSR 303 R	CCTGCAGATAAAGTCGCTAT			

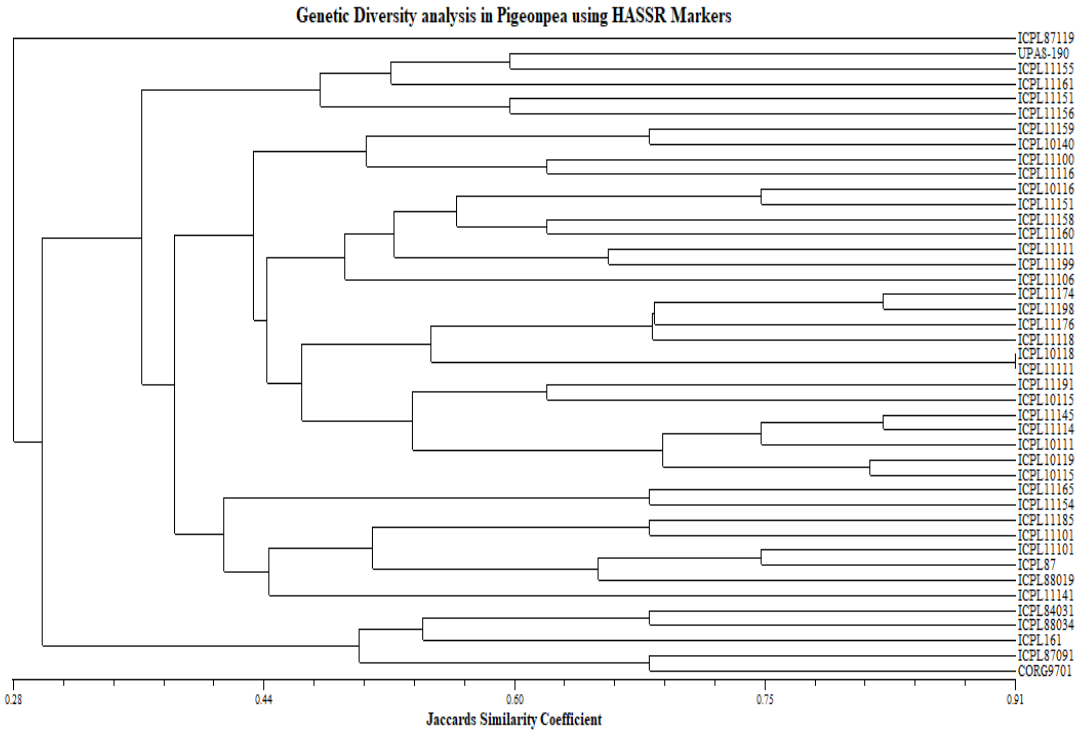

Fig 2: Cluster analysis of 43 pigeonpea genotypes based on UPGMA analysis.

CONCLUSION

In the present investigation, we have successfully assessed the levels of inter and intraspecific diversity relationships among 43 exra early pigeonpea genotypes. Pigeonpea genotype ICPL87119 is wilt resistant and also genetically more distant from other pigeonpea genotypes *viz*., ICPL84031, ICPL88014, ICPL161, ICPL87091 and CORG9701. Hybridization among these diverse parents may helpful to obtain better extra early genotypes with wilt resistance. Results obtained from the present investigation would be highly useful in Pigeonpea breeding programs and may be used for further crop improvement using advance marker systems.

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