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M Dhanushasree
PG Scholar, Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

A Thanga Hemavathy
Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

D Malarvizhi
Department of Plant Genetic Resources, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

S Kavitha
Department of Plant Genetic Resources, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Corresponding Author:
M Dhanushasree
PG Scholar, Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Molecular genetic diversity analysis of pigeonpea germplasm using microsatellite markers

M Dhanushasree, A Thanga Hemavathy, D Malarvizhi and S Kavitha

Abstract
Molecular diversity of fifty five pigeonpea genotypes were studied with fifteen microsatellite markers. Among them, four markers produced polymorphic bands and six markers were identified as monomorphic. The PIC value of these polymorphic markers ranged between 0.07 to 0.36. Marker CcM1251 (0.36) recorded the maximum PIC value and the minimum PIC value for the markers CcM0494 and CCac010 (0.07). Molecular tree constructed using Ordinal Neighbour Joining analysis grouped the fifty five genotypes into six major clusters. Among the six clusters, Cluster I was the largest with forty genotypes followed by cluster VIII (10), cluster VI (9), cluster II (5) and clusters V and VII (4). Cluster III and IV were the monogenic clusters with one genotype in each cluster. The study concludes that 38 percent of genotypes present in cluster I which signifies lack of divergence among the genotypes, while the genotypes *viz.*, ICPL 11301 and CRG 16-07 were identified to be more diverse forms solitary cluster which can be utilized as parents for heterosis breeding programmes.

Keywords: Microsatellites, polymorphism information content, amplification, molecular diversity

Introduction
Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is one of the multipurpose perennial legume crops mainly cultivated in tropical and subtropical regions of the world. An idea of diversity in pigeonpea germplasm collections is crucial for any plant breeding program. Morphological markers are easily influenced by environment and accuracy of their estimation is also questionable which paved the way for utilization of molecular markers. Diversity analysis was carried out by using powerful DNA based molecular markers. However, most agronomic traits in pigeonpea remains unmapped at molecular level due to the low level of DNA polymorphism and inadequate number of validated molecular markers. Therefore, SSR markers are ideal for studying the genetic diversity (Kimaro *et al.*, 2020) [4]. SSRs or microsatellites, known as neutral markers were chosen because of high polymorphism, detection of variation in multiple alleles, co-dominance, and reproducibility, easily detected by polymerase chain reaction (PCR), relatively abundant and located throughout the genome. The current research aim was to assess the molecular diversity and relationship among fifty five genotypes of pigeonpea using fifteen microsatellites.

Materials
Plant material
The study material consisted of fifty five pigeonpea genotypes maintained at Department of Pulses, Tamil Nadu Agricultural University, and Coimbatore. Fresh leaf samples were collected from fifteen days old seedlings for DNA Extraction.

Isolation of DNA
DNA isolation was carried out in Center for Excellence in Molecular Biology (CEMB), Tamil Nadu Agricultural University, and Coimbatore by following modified CTAB ((Cetyl Trimethyl Ammonium Bromide) method (Thompson and Murray, 1980) [5]. The quality check and concentration of the DNA were checked by using 0.8% agarose, then the gel was allowed to run for 30 minutes at 120 V in gel electrophoresis unit.

Polymerase Chain Reaction (PCR)
Fifteen pigeonpea specific microsatellites were used for diversity studies. The PCR reaction mixture was prepared and amplification was carried out in Thermo cycler. The following thermal cycling were used as follows: initial denaturation at 94 °C for 1 min, followed by 35

cycles of denaturation at 94 °C for 1 min, annealing (56-72 °C) for 1 min, 72 °C for 1 min and final primer extension at 72 °C for 20 minutes. The product obtained after thermo cycling was subjected to gel electrophoresis. The amplified reaction mixture was loaded on % agarose gel stained with 3% ethidium bromide. The gel electrophoresis unit was allowed to run for 2 hours at 110 V with reference to 100 bp ladder. Using BIO-RAD gel documentation system, the gel was photographed using Trans illuminating UV light.

Data analysis

Visual scoring of microsatellite alleles was done with reference to 100 bp ladder. '1' (Presence) and '0' (Absent) scoring were made for alleles of each primer. Data analysis was carried out using Darwin software package. Polymorphism Information Content (PIC) value was recorded for all microsatellites which is used to measure the capacity of the marker to detect polymorphic loci among genotypes. The formula for PIC value was as follows PIC $=1-\Sigma pi^2$, where, pi is the frequency of the 'i' th allele (Devi and Jayamani, 2020) [2]. Cluster analysis and tree construction were carried out with Ordinal Neighbour Joining using Darwin 6.0.2.1 software.

Results and Discussion

Molecular diversity studies were carried out for fifty five pigeonpea genotypes. (Table 1) by using fifteen microsatellites. Out of which, five markers failed to amplify for various genotypes. Only ten markers showed amplification for all genotypes listed in the Table 2. Four markers *viz.*, CcM0494, CcM1251, CCac003 and CCac010 were identified to be polymorphic, whereas six markers showed homomorphism. Among the polymorphic markers, all markers detected to have two alleles. The allele size for these markers ranged between 150-210 bp with an average of two alleles per marker.

The genotypes were visually scored with reference to 100 bp ladder. Usually, range of Polymorphism Information Content (PIC) was 1 and 0 for polymorphic and monomorphic markers, respectively. Polymorphism information content (PIC) value ranged between 0.07 to 0.36. The maximum PIC value was recorded for marker CcM1251 and the minimum PIC value for the markers CcM0494 and CCac010. The results were in analogy with the results of Hemavathy *et al*. (2017) [3] with minimum range of PIC value for the marker CcM1381 of 0.033. Marker CcM1251 was found to be polymorphic generated bands at different two different base pairs were given in the Plate.1.

Cluster analysis of pigeonpea genotypes using ten microsatellites resulted in the formation of tree cluster (Fig.1) which grouped fifty five pigeonpea genotypes into eight major clusters (Table 3). The above results were supported by the following findings of Naing *et al*. (2021) [9] and Thenmozhi *et al*. (2022) [8] classified fifty pigeonpea genotypes into six major clusters and forty eight pigeonpea genotypes into seven clusters, respectively. Similarly, Addae-Frimpomaah *et al*. (2021) [1] stated that six polymorphic SSRs were used for molecular diversity analysis of thirty two pigeonpea genotypes resulted in the formation of four major clusters with mean PIC value of 0.25. According to Reddy *et al*. (2022) [7], thirty two genotypes were clustered by using fifteen polymorphic markers resulted in the formation of ten major clusters with an average PIC value of 0.19 for each marker. Cluster I had the maximum number of genotypes (21), followed by cluster VIII (10), cluster VI (9), cluster II (5) and clusters V and VII (4). Cluster III and cluster IV were the solitary clusters with one genotype in each cluster. ICPL 11301 and CRG 16-07 were the solitary genotypes in clusters III and IV, respectively. Similarly, the monogenic clusters were reported by Hemavathy *et al*. (2017) [3] and Thenmozhi *et al*. (2022) [8]. Cluster analysis signified that divergence among the genotypes was low as 38 percent of the genotypes were present cluster I. Genotypes *viz.*, ICPL 11301 and CRG 16-07 were identified to be more diverse found in solitary clusters. Therefore, based on the parse performance, the genotypes with good yield attributes were selected as parents for future crop improvement programmes. More genetic divergence was present between genotypes from different clusters than those in the same cluster.

Table 1: List of genotypes used for molecular diversity analysis

S. No.	Genotypes	Origin
1	CRG 16-12	TNAU, Coimbatore
2	CRG 16-07	TNAU, Coimbatore
3	ICPL 20325	ICRISAT, Telangana
4	ICPL 11301	ICRISAT, Telangana
5	ICP 9808	ICRISAT, Telangana
6	ICP 91	ICRISAT, Telangana
7	ICP 2391	ICRISAT, Telangana
8	IC 525443	ICRISAT, Telangana
9	IC 525520	ICRISAT, Telangana
10	IC 342747	ICRISAT, Telangana
11	IC 73895	ICRISAT, Telangana
12	ACP 1225	IIPR, Kanpur
13	AL 1692	PAU, Ludhiana
14	AL 1727	PAU, Ludhiana
15	C 2542	IIPR, Kanpur
16	DPP-2-183	IIPR, Kanpur
17	DPP-2-188	IIPR, Kanpur
18	DPP-2-89	IIPR, Kanpur
19	DPP-3-2	IIPR, Kanpur
20	PA 509	GBPAU&T, Pantnagar
21	RVKT 333	IIPR, Kanpur
22	IPAE 18-04	IIPR, Kanpur

23	PA 21-45	Pusa, New Delhi
24	PA 21-14	Pusa, New Delhi
25	PA 21-27	Pusa, New Delhi
26	PA 21-29	Pusa, New Delhi
27	PAU 881	PAU, Ludhiana
28	IPAE 15-08	IIPR, Kanpur
29	PA 21-61	Pusa, New Delhi
30	AL 2324	PAU, Ludhiana
31	TJT 501	BARC & Khargone
32	BDN 711	ARS, Badnapur
33	BWR 243	IIPR, Kanpur
34	BWR 853	IIPR, Kanpur
35	UPAS 120	GBPAU&T, Pantnagar
36	BWR 253	IIPR, Kanpur
37	BWR 553	IIPR, Kanpur
38	BWR 23	IIPR, Kanpur
39	BWR 316	IIPR, Kanpur
40	BSMR 65	IIPR, Kanpur
41	BSMR 399	IIPR, Kanpur
42	BWR 164	IIPR, Kanpur
43	BWR 153	IIPR, Kanpur
44	BWR 134	IIPR, Kanpur
45	IC 339057	ICRISAT, Telangana
46	IC 525468	ICRISAT, Telangana
47	AL 1736	PAU, Ludhiana
48	AL 1739	PAU, Ludhiana
49	C 11	ICRISAT, Telangana
50	BSMR 26	IIPR, Kanpur
51	BSMR 1	IIPR, Kanpur
52	Co(Rg) 7	TNAU, Coimbatore
53	VBN 1	NPRC, Vamban
54	APK 1	RRS, Aruppukkottai
55	VLA 1	ICRISAT, Telangana

Table 2: List of microsatellite markers used for molecular diversity analysis

Primer Name	Orientation	Primer Sequence	Primer Sequence Length	Allele size range (bp)	Number of alleles	PIC Value
CcM0494	F	ACGTGAAAAATCCGCAACTT	20	150-170	2	0.07
	R	GCTTGTGTTTCAAAATCCAACTT	23			
CcM1251	F	CAAATGGCAGAACAGAGCAG	20	200-210	2	0.36
	R	CGGAGATTGCATTGTTCCTT	20			
CCac003	F	TGCTTCAAGTTGCCTACCAG	20	170-180	2	0.25
	R	TCAAGGGAGGTGGACTACAAA	21			
CCac010	F	GATAGCACACACACACACAACA	22	200-230	2	0.07
	R	TACCTTAGGGTCACCAACGA	20			
CCac020	F	GGGAAACAAAATATCCCCTAATC	23	300	1	0
	R	TAATCACACACATCACACCTAGCA	24			
CcM0121	F	AGAAATTGGAGGCTTGGTCA	20	300	1	0
	R	GGTATAAGGCTCAAACCCGA	20			
CcM0444	F	TGTCATGAGTGGCTGATCCT	20	190	1	0
	R	TCAACCAAAATCCAAACCAA	20			
CcM2097	F	TGATAGGAATATTTCGGCGG	20	170	1	0
	R	CCTTTGAAATTGAAGGCGAG	20			
CCac013	F	GTGAGTGAGAGTGAGTGTATTTGTG	25	210	1	0
	R	GCTCTGATGCCAAATGTTGA	20			
CCac012	F	ACCTTGCTTGTTTCGCTTTT	20	190	1	0
	R	AAGGGAGGTGGACTACAAGGA	21			

Table 3: Cluster composition of pigeonpea genotypes for molecular diversity

Clusters	No of Genotypes	Name of Genotypes
I	21	ICP 9808, ICP 91, IC 525520, IC 73895, CO(Rg) 7, BWR 153, AL 1727, C 2542, BWR 553, AL 2324, BWR 243, PAU 881, PA 21-14, PA 21-45, PA 21-27, DPP-3-2, IPAE 18-04, DPP-2-188, AL 1739, UPAS 120, RVKT 333
II	5	ICPL 20325, PA 21-61, BDN 711, BWR 23, APK 1
III	1	ICPL 11301
IV	1	CRG 16-07
V	4	PA 509, BSMR 1, VBN 1, TJT 501

VI	9	BWR 316, BSMR 65, BSMR 399, BWR 134, IC 525468, AL 1736, C 11, BWR 253, VLA 1
VII	4	CRG 16-12, ICP 2391, DPP-2-89, BWR 853
VIII	10	IC 525443, IC 342747, ACP 1225, AL 1692, DPP-2-183, BWR 164, IC 339057, PA 21-29, IPAE 15-08, BSMR 26

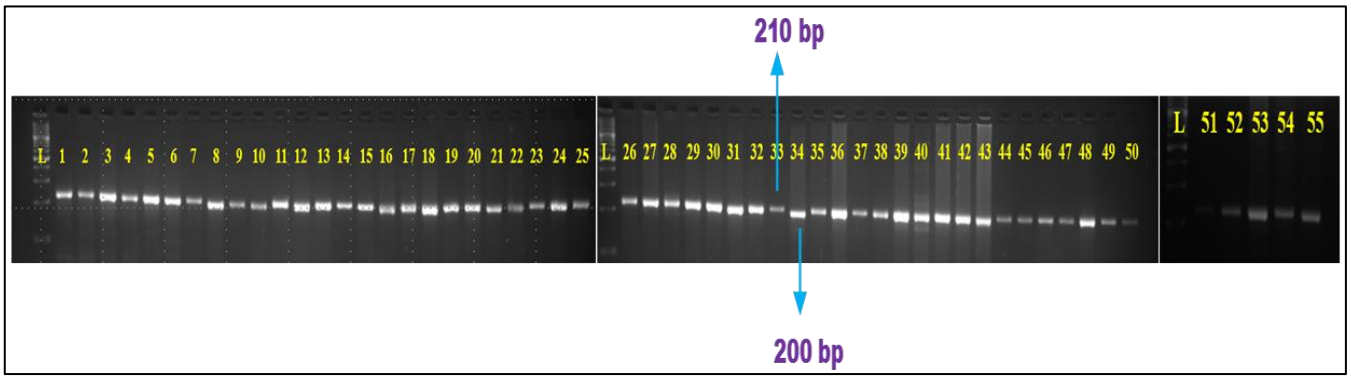

Plate 1: Profile generated by SSR CcM1251 showed polymorphism at 200 and 210 bp

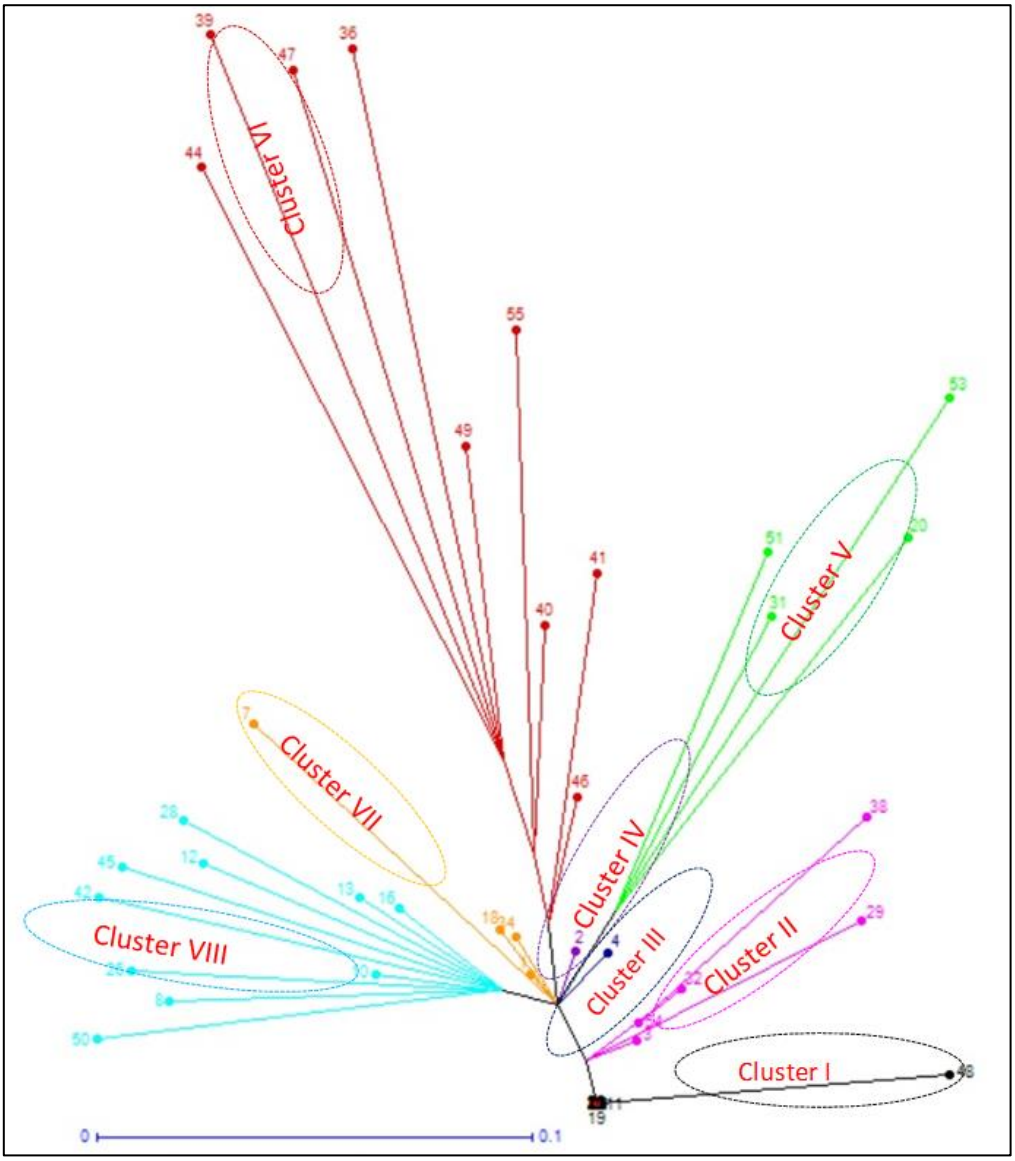

Fig 1: Tree construction for molecular clustering of pigeonpea genotypes using 10 SSR primers

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