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Genetic Diversity among Pigeonpea (*Cajanus cajan* L. Millsp.) Genotypes Using Genic SSRs with Putative Function for Drought Tolerance

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One hundred and thirty eight pigeonpea genotypes were analyzed for molecular genetic diversity using 34 SSR markers with putative function for drought tolerance. The study revealed considerable molecular genetic diversity among genotypes. Fifty two alleles were obtained with 34 SSR markers while, 1 to 3 alleles was scored with an average of ~1.6 alleles for each SSR. Three alleles were amplified by markers ASSR1, ASSR93 and ASSR97. Of these, 15 SSR markers were found to be polymorphic which identified 33 alleles among 138 genotypes. The average PIC value of these polymorphic SSRs was 0.22 with a range of 0.01 for ASSR308 to 0.38 in ASSR97. Significant positive correlation was observed between PIC values with number of alleles amplified per primer ($r = 0.58^*$, $P < 0.05$), and gene diversity ($r = 0.99^{**}$, $P < 0.01$) and between allele number and gene diversity ($r = 0.57^*$, $P<0.05$). The average genetic distance for all pair wise comparisons was estimated as 0.27. The highest genetic distance of 0.69 was recorded between genotypes PAU-881 and LRG-41. Cluster analysis, done by UPGMA following Nei's similarity matrix and population structure analyses grouped 138 pigeonpea genotypes into seven sub-populations.

Introduction

Pigeonpea (*Cajanus cajan* L. Millsp.) is predominantly a rainfed crop grown across the world. Although it is considered as a drought tolerant crop among all grain legumes and largely grown under rainfed conditions (Keller and Ludlow, 1993) across the world, productivity is highly affected by drought if it coincide with flowering and early pod development stages (Lopez *et al.*, 1997). There is large variation for days to maturity, ranging from extra early (90 days) to very long (300 days) among available pigeonpea germplasm. The intermittent periods of drought can affect the growth and yield of specially short-duration pigeonpea sown at the start of the rainy season. As pigeonpea is cultivated under rain-fed conditions, occurrence of drought may be episodic in varying degrees in the majority of the growing season in dry land agricultural systems. An increase in temperature above 2.5°C, is known to convey negative effects on global agriculture on the whole. Adverse impact of drought on crop growth and development causes yield reduction. Despite several decades of intensive efforts in

different crop improvement programmes, the yield level reached a plateau, owing to the narrow genetic base and conventional breeding procedures. The high degree of complexity associated with the genetic enhancement through breeding procedures can be successfully overcome by the employment of biotechnological interventions (Chakravarthy and Negi, 2014).

The recent advancement in pigeonpea genomic resources resulted in the development of molecular markers, genetic maps, transcriptomic or genome sequence required for molecular breeding. Discovery of molecular markers led to genetic diversity analysis using restriction fragment length polymorphism (Sivaramakrishnan *et al.,* 2002), amplified fragment length polymorphism (Panguluri *et al.,* 2006), random amplification of polymorphic DNA (Yadav *et al.,* 2012), microsatellite markers (Singh *et al.,* 2013) and DArT (Yang *et al.,* 2006). Nevertheless, the molecular basis of most agronomic traits in pigeonpea remains unexplored due to the low level of DNA polymorphism and limited number of validated molecular markers. The presence of genetic diversity plays a vital role for a successful breeding program. Genetic diversity is essential prerequisite in breeding for drought tolerance, increased yields, wider adaptation and desirable quality. Earlier studies on genetic diversity with limited number of genotypes has been reported in pigeonpea *viz*., 36 elite cultivated genotypes (Singh *et al.,* 2013), 45 genotypes (Datta *et al.,* 2013), 16 cultivars and 2 wild relatives (Yadav *et al.,* 2012), 15 genotypes (Shende and Raut, 2013), 49 genotypes (Rekha *et al.,* 2011), 88 accessions (Songok *et al.,* 2010), 16 genotypes (Singh *et al.,* 2008) and 14 genotypes (Chakraborty *et al.,* 2013). These studies however, focused on studying overall genetic diversity among pigeonpea germplasm using genic and genomic SSRs not specifically SSR markers related to drought tolerance.

The objective of the present investigation was to study the level of molecular genetic diversity and population structure among pigeonpea cultivars and germplasm collection using genic SSR markers linked with putative function for drought tolerance.

Materials and Methods

Plant material

A total of one hundred and thirty eight pigeonpea genotypes, adapted to different climatic conditions, were received from Indian Institute of Pulses Research, Kanpur, India. Information on sources of origin of these genotypes is given in Online Resource 1. The genotypes included in the study are mostly the released varieties for different production areas in India; advanced breeding lines and germplasm accessions from Regional Research Station, National Bureau of Plant Genetic Resources, Hyderabad. All these genotypes were sown in two rows of 2.5m plot, with a row to row spacing of 90 cm and plant to plant spacing of 30 cm in augmented block design at CRIDA, Hyderabad. The recommended fertilizer doses and agronomic operations were carried out for adequate protection against pests, diseases and weeds.

DNA extraction and PCR

Genomic DNA was extracted following CTAB method (Paterson *et al.,* 1993) with minor modifications from top most fully expanded leaf samples of four-week old plants for each genotype. Thirty four genic SSR markers previously reported by Dutta *et al.,* (2011) were used to amplify the DNA for genotyping. The PCR reaction contained 1.0 unit of Taq DNA polymerase, 1X Taq buffer

and 200µM of each dNTP. Approximately, 50ng of genomic DNA and 10 picomoles of each primer were used and the volume was made up to 20µl using sterile distilled water. DNA amplification was carried out in a Thermal Cycler (Applied Biosystems) with a PCR profile comprised an initial denaturation for 5 min at 94°C followed by 35 cycles with a denaturing step at 94°C for 45 seconds, a primer annealing at 60°C for 45 seconds and an extension at 72°C for 45 seconds. After the last cycle, a final extension was carried out at 72°C for 5 min. Amplified PCR products were resolved through electrophoresis at 80 volts for one hour and 30 minutes in 4% agarose gel containing 0.5µg/ml ethidium bromide and photographed under ultraviolet light with Vilber Loumat gel documentation system. The SSR amplification profiles were scored based on the size (bp) of the amplicons obtained among 138 genotypes using Biovision Software, USA.

Statistical analyses

Gene diversity, heterozygosity and polymorphism information content (PIC) for each of the primer pair was calculated using Power Marker v.3.25 software (Liu and Muse, 2005). Genetic distances between the genotypes were also calculated (Nei, 1973). Phylogenetic tree was constructed using UPGMA (unweighted pair-group method using arithmetic average) by neighbor-joining method and dendogram was generated by MEGA software version 5.0 (Tamura *et al.,* 2011). The STRUCTURE 2.3.4 (Pritchard *et al.,* 2000; Falush *et al.,* 2003) software was used to detect population structure and assign individuals to subpopulations following model based on clustering by Bayesian approach which identifies clusters based on a fit to Hardy–Weinberg linkage equilibrium. The population structure analysis was used to infer historical lineages that show grouping of similar genotypes. For each cluster K, five replications were run where each run was implemented with a burn-in period of 100,000 steps followed by 100,000 Monte Carlo Markov Chain replicates derived for each K and then plotted to find the plateau of the ΔK values (Evanno *et al.,* 2005).

Results and Discussion

Research for development of drought tolerant crops is of urgent priority, as water stress is one of the main reasons for the major crop losses globally and is expected to exacerbate due to projected climate change impacts. Pigeonpea being an important source of dietary protein and a major legume in the arid and semi-arid regions, may be adversely affected due to climate change unless efforts made to develop tolerant cultivars. Identification of diverse parents is essentially required before carrying out successful crop improvement program (Tidke and Ranawade, 2017). In this section we discuss the results pertaining to molecular characterization of 138 pigeonpea genotypes adapted to diverse climatic conditions using SSR markers associated with putative function for drought tolerance.

Polymorphism and marker efficiency

Pigeonpea genotypes characterized using 34 SSR markers revealed 15 SSRs as polymorphic (~44%) while 19 as monomorphic (~66%). The polymorphic SSRs was used to examine the degree of genetic variation among pigeonpea genotypes. List of polymorphic SSRs and their predicted function of genes linked with these SSRs, amplicon size was given in Table 1. A total of fifty two alleles were obtained with 34 SSR markers and number of alleles scored for each SSR loci ranged from 1 to 3 with an average of ~1.6 alleles per primer pair. Three alleles were amplified by ASSR1, ASSR93 and ASSR97. The amplification profile of

ASSR93 was given in Fig. 1. The PIC value of SSRs ranged from 0.01 (ASSR308) to 0.38 (ASSR97) with an average being 0.22 (Table 2). Among these 15 polymorphic SSR markers, 8 SSRs *viz*., ASSR1, ASSR3, ASSR8, ASSR19, ASSR93, ASSR97, ASSR280 and ASSR648 gave PIC >0.25 with an average of 0.32 and 19 alleles with an average of 2.38 alleles/primer. The major allele frequencies among the primers tested varied between 0.59 (ASSR19) to 0.99 (ASSR213) with an average of 0.82. On the other hand, gene diversity varied from 0.01 to 0.48 with an average of 0.26.

The study revealed a total of 52 alleles using 34 SSR markers among 138 genotypes which was similar to findings made by Singh *et al.*, (2013), who reported 59 alleles using 60 SSR markers among 36 genotypes. The PIC of SSRs obtained in our study was relatively higher than those reported by Khalekar *et al.*, (2014) and Datta *et al.*, (2013). Several other workers also reported different level of genetic diversity in pigeonpea (Panguluri *et al.*, 2006; Yang *et al.*, 2006; Singh *et al.*, 2013). This variation in genetic diversity is probably attributed to diversification in morphology, use of common ancestors for the development of new cultivars (Panguluri *et al.*, 2006; Yang *et al.*, 2006). The present study also revealed significant positive correlation between PIC values with number of alleles amplified per primer ($r = 0.58^*$, $P < 0.05$) and gene diversity ($r = 0.99^{**}$, $P < 0.01$), and between allele number and gene diversity ($r = 0.57^*$, $P<0.05$).

Genetic similarity among genotypes

Genetic distance among the 138 pigeonpea genotypes was calculated to identify the relatedness between genotypes. The genetic distance measured through polymorphic SSRs revealed varying degree of genetic relatedness among the pigeonpea genotypes. The average genetic distance for all pair wise comparisons was 0.27. The highest genetic distance of 0.69 was recorded between genotypes PAU-881 and LRG-41; PT-00-022 and LRG-41, followed by BWR-153 and RVK-281; RJR-292 and GT-1 which exhibited genetic distance of 0.67. Whereas, 10 genotype combinations *viz*., CO-6 and AL-1578, GT-100 and AL-1578, ICP-84031 and AL-1578, UPAS-120 and LRG-41, AL-1816 and RVK-278, VKG-14151 and RVK-281, RVK-278 and AL-1578, PG-12 and AL-1816, Pusa-84 and CO-5, Pusa-84 and GRG-2761 had genetic distance of 0.64.

Cluster analysis

The cluster analysis based on Power marker software using polymorphic SSR markers resulted in separation of the genotypes into two major clusters (Fig. 2). Cluster II was larger comprising of 97 genotypes. Further, this cluster was sub-divided into five sub-clusters with number of genotypes per cluster ranging from 6 to 35. On the other hand, cluster I is divided into two sub-clusters, consisting of 31 and 10 genotypes respectively.

Population structure analysis

Population structure analysis divided 138 pigeonpea genotypes into seven different groups, assuming low levels of admixture between subpopulations (Fig. 3 and Table 3). List of pigeonpea genotypes corresponding to different groups is given in Online Resource 2. The number of genotypes ranged from 15 in group G_6 to 29 in group G_2. Groups G_1 and G_3 comprised of 17 genotypes each whereas, groups G_5 and G_7 contained 19 genotypes each. On the other hand group G_4 had 22 genotypes. Among the genotypes tested, higher gene diversity was displayed within G_6 (0.25) followed by G_4 (0.23) and G_1 (0.22), whereas a low level (0.06) of gene diversity was displayed by G_7 (Table 4).

Table.1 Details of 34 SSRs used in the present study and predicted function of their genes

Sl. No.	SSR marker	SSR motif	Predicted function	Amplicon Size (bp)
1	ASSR-1	(GA)10	Putative Kinase	100-120
2	ASSR-3	(AGAAAG)5	Cytochrome P450 Possessing cinnamate 4-hydroxylase activity	130-150
3	ASSR-8	(AGA)9	Cu/Zn-Superoxide dismutase (SOD)	140-150
4	ASSR-19	(TGTTCA)5	DNA binding protein (Homeodomain)	150-160
5	ASSR-23	(CCTTCT)5	Acetyltransferase	150-170
6	ASSR-25	(GA)10	Ser/Thr protein kinase	180
7	ASSR-36	(TC)14	Global transcription factor group	160
8	ASSR-39	(GAA)7	Cyclin	180
9	ASSR-49	(TC)10	calmodulin binding protein	180
10	ASSR-66	(CT)12	Hypothetical protein	180
11	ASSR-70	(GGTAGA)6	Gamma glutamylcyclotransferase	170-200
12	ASSR-91	(GGTTA)5	Hypothetical protein	120
13	ASSR-93	(CATTTG)5	Hypothetical protein	160-180
14	ASSR-97	(ATGGAC)8	Chloroplast targeted copper chaperone	150-190
15	ASSR-121	(TCT)8	Ethylene responsive transcription factor	180
16	ASSR-138	(CTT)8	r2r3-myb transcription factor	160
17	ASSR-148	(CAA)7	Ethylene-responsive transcription factor	110-120
18	ASSR-163	(TCA)8	Heat shock protein binding	210
19	ASSR-168	(TCA)9	Heat shock protein	150-160
20	ASSR-213	(AGG)7	Mitogen-activated protein kinase 1	150-160
21	ASSR-275	(TAAT)5	MYB transcription factor MYB48	130
22	ASSR-279	(ACAGGA)7	Senescence-inducible chloroplast stay-green protein-1	180-190
23	ASSR-280	(TGGCAT)5	Senescence-inducible chloroplast stay-green protein	160-170
24	ASSR-304	(GTT)7	Ethylene responsive transcription factor	110
25	ASSR-308	(TC)10	Serine/threonine protein kinase	150-160
26	ASSR-388	(CCA)7	No homology	150
27	ASSR-538	(TC)9	MYB transcription factor MYB34	150
28	ASSR-609	(ACC)6	Leucine Rich family protein	190
29	ASSR-648	(GAT)6	Protein of early response to dehydration	150-160
30	ASSR-973	(TTG)6	Ethylene insensitive protein	150
31	ASSR-1092	(CGG)6	Serine/threonine protein kinase catalytic domain	160
32	ASSR-1214	(ACA)6	WRKY family transcription factor	170
33	ASSR-1217	(GGA)6	WRKY family transcription factor	190
34	ASSR-1639	(AAT)6	Senescence-associated protein	150

Table.2 Number of alleles, gene diversity and PIC of polymorphic microsatellite markers

Sl. No.	Marker	Number of Alleles	Amplicon Size (bp)	Gene Diversity	PIC	Major allele frequency
1	ASSR1	3	100-120	0.39	0.33	0.75
2	ASSR3	2	130-150	0.31	0.26	0.81
3	ASSR8	2	140-150	0.29	0.25	0.83
4	ASSR19	2	150-160	0.48	0.37	0.59
5	ASSR23	2	150-170	0.28	0.24	0.83
6	ASSR70	2	170-200	0.20	0.18	0.89
7	ASSR93	3	160-180	0.45	0.38	0.69
8	ASSR97	3	150-190	0.48	0.38	0.62
9	ASSR148	2	110-120	0.22	0.20	0.87
10	ASSR168	2	150-160	0.12	0.11	0.94
11	ASSR213	2	150-160	0.01	0.01	0.99
12	ASSR279	2	180-190	0.04	0.04	0.98
13	ASSR280	2	160-170	0.38	0.31	0.75
14	ASSR308	2	150-160	0.01	0.01	0.99
15	ASSR648	2	150-160	0.31	0.26	0.81
	Average	2.20		0.26	0.22	0.82

Fig.1 PCR amplification pattern of 138 pigeonpea genotypes using ASSR93 primers. M100=100bp DNA size marker

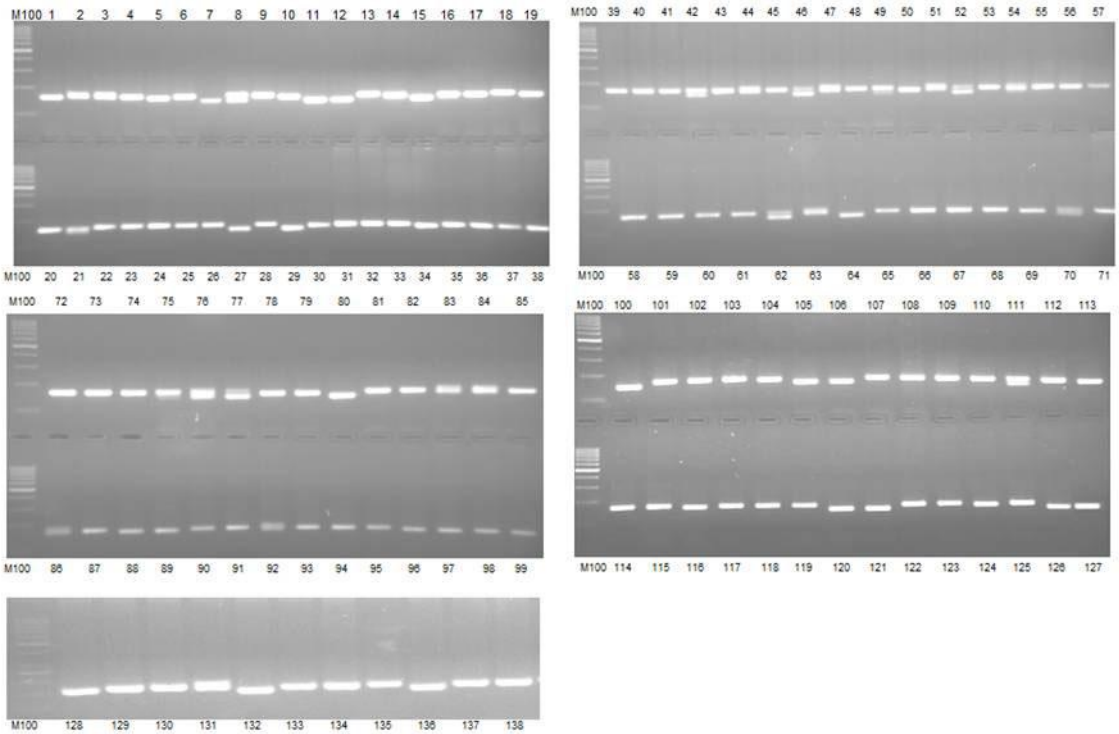


Table.3 list of pigeonpea genotypes corresponding to a group in population structure analysis G_1: 54-63; G_2: 105-44; G_3: 88-116; G_4: 129-4; G_5: 22-114; G_6: 134-123; G_7: 33-118

G_1		G_2		G_3		G_4		G_5		G_6		G_7	
54	ICP84031	105	RVK272	88	Pusa855	129	TS3R	22	BDN708	134	Vipula	33	C11
55	ICPL151	17	BDN2	66	LRG30	138	WRP1	85	PT221	130	TV1	136	WRG27
57	ICPL87	61	Jamadhar local	60	JA4	25	BPG512	117	RVK286	35	CO5	132	Vamban1
87	Pusa84	26	BPG513	68	LRG41	34	Chaple	42	GRG2006	9	AL1816	48	GS1
50	GT100	120	SNJ201151	56	ICPL85063	51	Gulyal red	40	GP101	19	BDN20086	93	RJR232
83	PT002251	101	RJR353	96	RJR292	62	JKM189	126	TAT9903	122	SNJ201187	2	AK101
81	PSRJ13147	31	BSMR853	72	NDA2	131	UPAS120	11	AL1855	73	Paras	91	RJR185
38	DA11	41	GRG0811	13	Amar	82	PT0022	6	AL15	70	Manak	1	AK022
52	HY3C	65	Karitogari	109	RVK277	74	PAU881	59	ICPL8863	89	Pusa991	58	ICPL88039
115	RVK283	24	BPG109	36	CO6	10	AL1817	3	AKP1	27	BRG1	90	RJR121
137	WRG53	97	RJR302	37	CORG9701	75	PBJ55C233	124	T1515	49	GT1	92	RJR223
133	Vamban2	119	SNJ2011103	39	GL1139	18	BDN200812	128	TS3	69	MA3	104	RJR81
64	K2	98	RJR314	127	TJT501	135	VKG14151	16	Banas	113	RVK281	102	RJR358
110	RVK278	80	PSR13229	47	GRG815	30	BSMR736	20	BDN20088	5	AKT9915	46	GRG333
77	PH12	79	PSR13227	8	AL1757	12	AL201	84	PT0431	123	SNJ201197	67	LRG38
76	PG12	108	RVK275	15	Azad	125	TAT10	45	GRG281			103	RJR67
63	JKM7	121	SNJ201171	116	RVK285	86	Pusa33	43	GRG20091			100	RJR33
		94	RJR246			7	AL1578	29	BSMR533			28	Brisha arhar
		21	BDN20089			71	NDA1	114	RVK282			118	SKM187
		112	RVK280			32	BWR153						
		99	RJR315			53	ICP13673						
		95	RJR263			4	AKT881						
		107	RVK274										
		23	Bennur local										
		111	RVK279										
		78	PRG158										
		14	Asha										
		106	RVK273										
		44	GRG2761										

Table.4 Summary statistics for the whole group of pigeonpea genotypes and subpopulations detected by structure analysis based on 15 polymorphic SSR markers

Statistics	Overall	G1	G2	G3	G4	G5	G6	G7
Sample size	138	17	29	17	22	19	15	19
Total number of alleles	33	25	24	25	27	22	28	19
Mean number of alleles	2.20	1.67	1.60	1.67	1.80	1.47	1.87	1.27
Major allele frequency	0.82	0.84	0.93	0.87	0.84	0.91	0.82	0.96
Gene diversity	0.26	0.22	0.10	0.19	0.23	0.13	0.25	0.06

Fig.2 UPGMA tree using Nei similarity coefficient

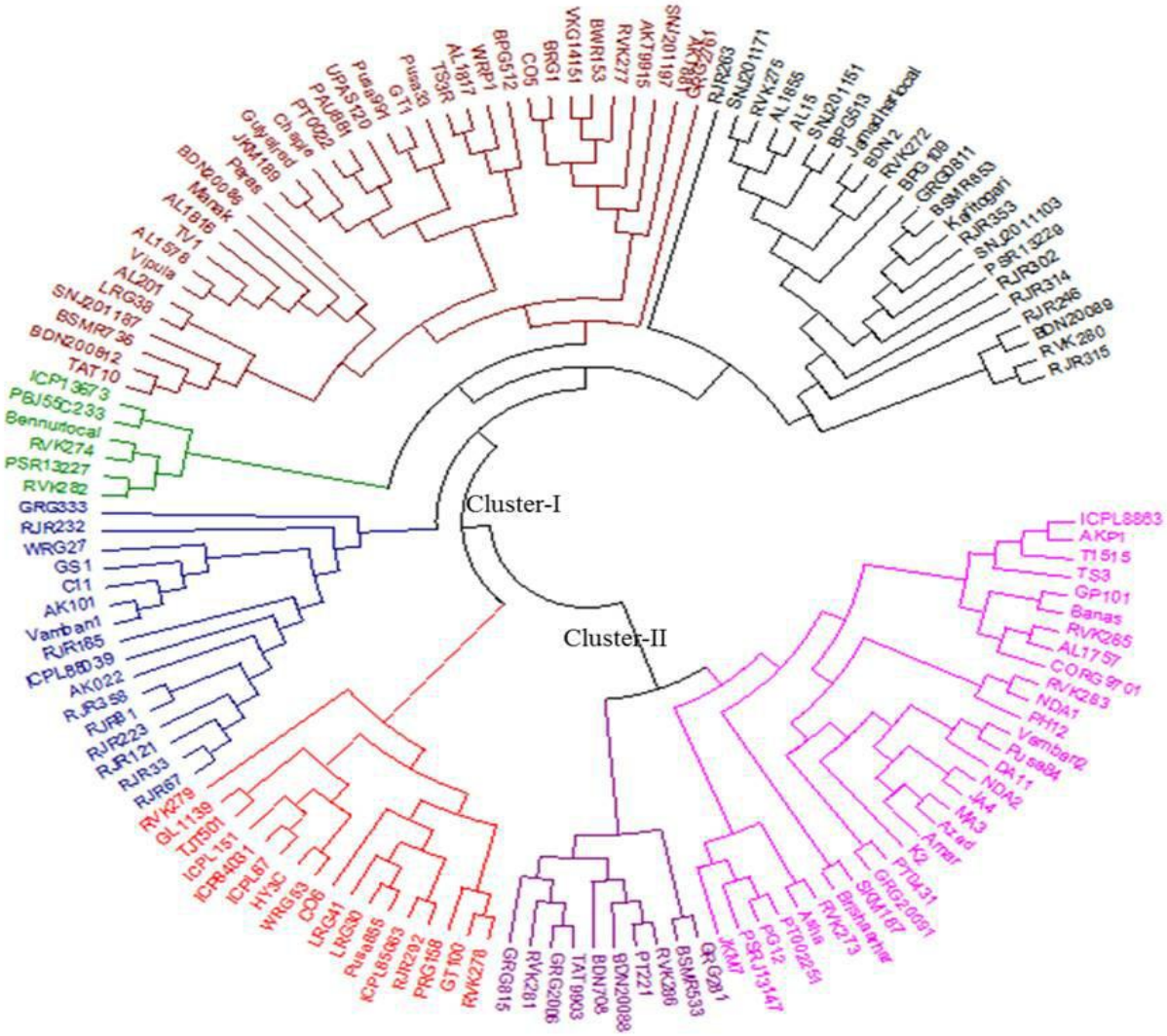
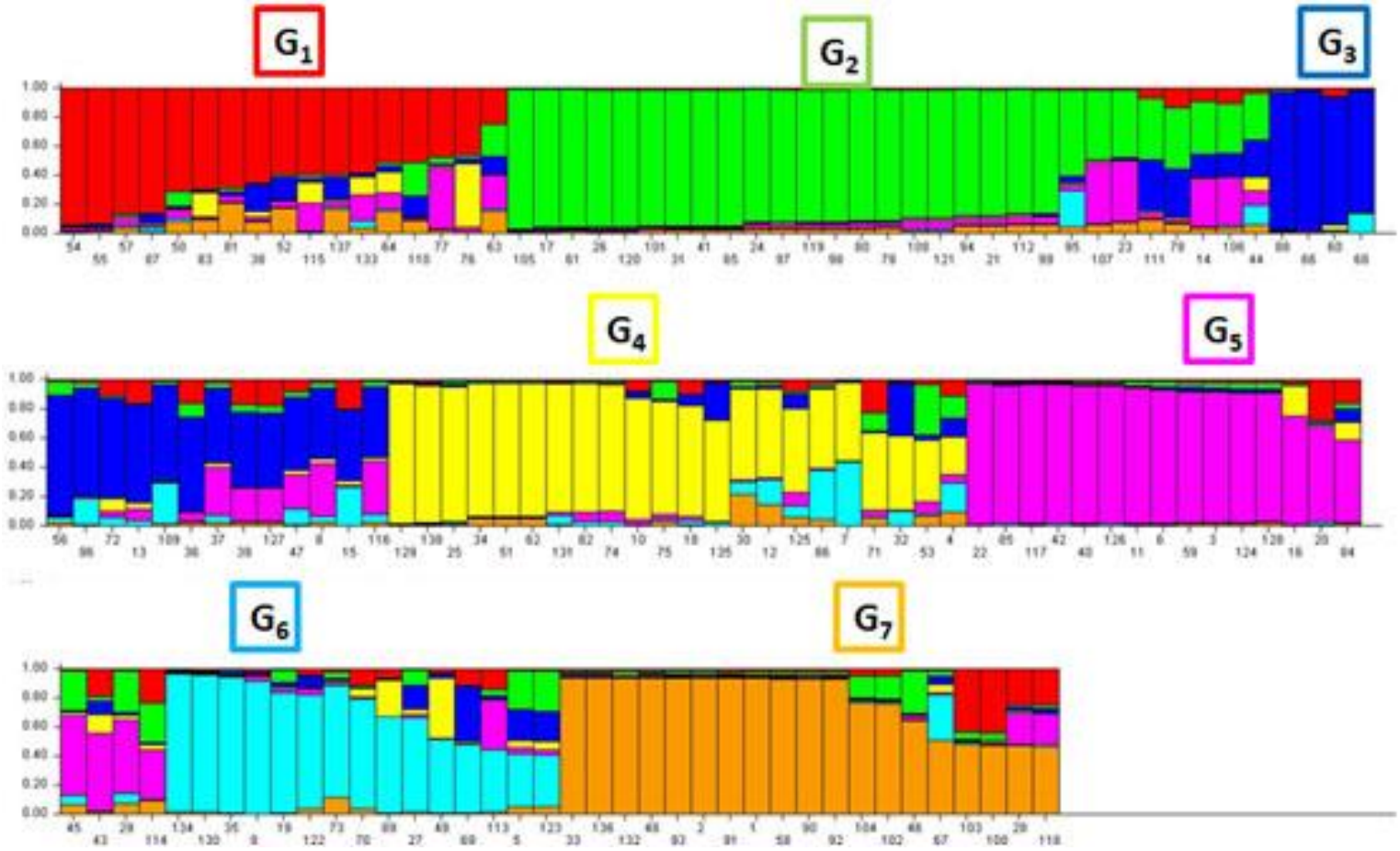


Fig.3 Population structure analysis. The y-axis is the subgroup membership, and the x-axis is the accessions. G (G_1 to G_7) stands for a subpopulation



Population structure analysis using SSR data revealed seven subpopulations, with varying degrees of admixture among subpopulations (Fig. 3). Structure analysis indicated the patterns of allele sharing among different pigeonpea genotypes from diverse agro-climatic regions and large scale sharing of alleles among the genotypes. In addition, UPGMA tree using neighbor joining also grouped the genotypes into seven subpopulations. The clusters found in structure analysis were almost consistent with the cluster analysis following UPGMA method using Nei similarity coefficient. Most of the genotypes were classified into the corresponding sub-population and branch was similar with a few exceptions.

Molecular analysis using drought linked genic SSR provided a good insight of genetic diversity and population structure among pigeonpea materials used in the present investigation. These findings will be useful in selection of diverse genotypes for development of new cultivars with adaptation to a broad range of environments. Further, the genotypes producing specific amplicons with SSR markers can be used for cultivar identification.

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