

Research Article

Assessment of Genetic Diversity among Pigeon pea Male Sterile lines and Popular Cultivars using SSR Markers

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Abstract

In the present study genetic diversity studies have been carried out using twenty five SSR markers in a set of pigeonpea genotypes comprising of male sterile lines and popular cultivars. The primers amplified a total of 99 bands with highest PIC of 0.79. The SSR markers grouped the genotypes into four clusters based on UPGMA and Principal Component Analysis. The male sterile lines along with PRG158 were grouped into three clusters while, the remaining cultivars formed a distinct cluster indicating the narrow genetic base of released cultivars. PCA revealed that phenotypic variation explained by the SSRs ranged from 4.7% to 49.6% with PC1 and PC2 accounting for 49.6% and 30.8% of total variation. The study indicated the existence of diversity between the male sterile lines and the popular cultivars which can be exploited for hybrid breeding program.

Key words

Pigeonpea, SSR markers, Genetic diversity, NTSYS-pc, PIC

Introduction

Pigeonpea or redgram is an important crop in India, where it is the second most important crop after chickpea. The early systematic studies of the genus *Cajanus* were based on morphological characters which have been shown to have limited genetic resolution especially at species levels (Van der Maesen, 1990 and Kimani, 2000). Earlier, morphological markers have been used for assessment of genetic diversity using cultivated pigeonpea and wild relatives (Greilhuber and Obermayer 1998). With the development of environmentally neutral, reliable and plant growth independent molecular markers, many researchers initiated the pigeonpea genetic diversity analysis. In the case of SSR markers, variations occur within non-coding sequences which have little or no effect on the organism's phenotype or function, but are still detectable at the DNA level and can be used as markers. SSR marker based technology is rapidly emerging as a highly promising marker system for DNA fingerprinting, analysis of genetic diversity and tagging of genes conferring agronomically useful characters.

Microsatellites or SSRs are stretches of tandemly arranged short sequence motifs which are abundant and highly polymorphic in several eukaryotic genomes. Assessment of genetic variability has been done using various molecular markers (Ratnaparkhe *et al.*, 1995 and Yadav *et al.*, 2010). Plant SSRs have been demonstrated to be a powerful tool in genotype identification and plant variety protection (Olufowote *et al.*, 1997), seed purity evaluation, germplasm conservation (Powell

et al., 1996), diversity studies (Xiao *et al.*, 1996), pedigree analysis and marker assisted selection (Yang *et al.*, 1994). Limited pools of pigeonpea germplasm have been characterized previously through RFLP (Sivaramakrishnan *et al.*, 2002), RAPD (Ratnaparkhe *et al.*, 1995), SSRs (Odeny *et al.*, 2007), AFLP (Panguluri *et al.*, 2006) and DaRT (Yang *et al.*, 2006).

Though India ranks first in area and production of pigeonpea, the average productivity of the crop is low. Exploitation of heterosis was considered as useful and should open vistas in pigeonpea production in India. The present study aims at assessment of genetic diversity among a set of superior, stable male sterile lines and popular varieties which can be the potential restorers for development of pigeonpea hybrids.

Materials and methods

Fifteen pigeonpea genotypes comprising of a set of stable male sterile lines from ICRISAT and released cultivars have been used for assessing genetic diversity using molecular markers. Pigeonpea genotypes were grown in pots and leaves from 30 days old seedlings were collected and frozen in liquid nitrogen and 100mg of leaf tissue was ground into fine powder and genomic DNA was extracted following CTAB procedure (Abdelnoor *et al.* 1995). The quality and quantity of DNA of the genotypes were checked through agarose gel electrophoresis (0.8%). A total of 26 SSRs were used for PCR amplification (Table 1). The PCR mixture comprised (10µl) of 25ng of genomic DNA, 250µM dNTPS, 1X PCR Buffer

with $MgCl_2$, 2.5pm of forward and reverse primers and 1 unit of Taq DNA Polymerase. Amplification was carried out in thermal cycler (Master Cycler, Eppendorf) by initial denaturation at $94^{\circ}C$ for 5 min followed by 35 cycles comprising of denaturation at $94^{\circ}C$ for 1min, optimum annealing temperature for 30 sec, primer extension at $72^{\circ}C$ for 30 sec and final extension of 7 min. The PCR products were resolved using 3% metaphor agarose and documented in gel documentation system (Alpha Innotech). A 100bp marker was used for approximate sizing of the fragments.

For each SSR marker, Polymorphism Information Content (PIC) was determined as described by senior et al., 1998. The amplified PCR products were scored as either presence (1) or absence (0) and entered in the form of a binary matrix. Jaccard's coefficient (J) (Jaccard, 1908) was used to calculate the genetic similarities (GS) based on SSR data.

The similarity matrix was analyzed using NTSYS-pc ver. 2.0 to produce an agglomerative hierarchical classification by employing UPGMA with average linkage. The mean of the similarity matrix would be the cut off line position on the dendrogram to identify the number of clusters. To test the goodness of fit of clustering to a set of data copheneic correlation or cophenetic value was estimated using the COPH and MXCOM options in NTSYS-pc program. Principle Component Analysis was carried out to confirm UPGMA based clustering using EIGEN module of NTSYS-pc.

Result and discussion

Estimation of Polymorphic Information Content: Identification and utilization of diverse germplasm is the central issue in plant breeding. More accurate and complete descriptions of elite breeding materials and understanding the patterns of genetic diversity could help determine future breeding strategies and facilitate introgression of diverse germplasm into the current genetic base, particularly from the view point of hybrid breeding. Among 26 SSR primers tested, 25 of them showing polymorphism were selected for further analysis. These primers resulted in the amplification of 99 bands, with the number of polymorphic bands per primer ranging from 2-5, the average being 4.18. Based on the PIC, the SSR primers PV20 and Ccac004 were most informative with value of 0.79. The least PIC value of 0.16 was depicted by SSR marker PP4 (Table 2). SSR banding profile using primer CCac036 is depicted in Fig. 1.

Estimation of Genetic Similarities: Genetic similarities analyzed using SSR data indicated varying degrees of genetic relatedness among the fifteen genotypes used in the study. Genetic

similarity coefficients between various pigeonpea genotypes were calculated from the SSR data matrix using Jaccard's similarity and the resulting GS matrix was further analysed using complete linkage clustering algorithm to depict genetic relationships (Table 3). Jaccard's similarity coefficients ranged from 0.06 to 0.73 depending on the diversity and pedigree of the genotypes used in the study. The similarity coefficients were highest between the genotypes ICPA 2189 and ICPA 2199 (0.73) followed by ICPA 2047-4 and ICPA 2048 (0.69). Minimum value of similarity coefficients were observed between ICPA 2043 and ICPA 2189 and ICPA 2051 and MRG 1004 (0.06) followed by ICPA 2043 and ICPA 2188 and ICPA 2043 and ICPA 2199; ICPA 2049 and MRG 1004 and ICPA 2078 and Laxmi (0.08). SSR markers associated with wilt resistance are extremely useful in screening the breeding material in the process of development of wilt resistant cultivars in absence of wilt sick plots (Singh *et al.*, 2013).

Clustering of pigeonpea genotypes: The dendrogram based on UPGMA clustering clearly divided the fifteen pigeonpea genotypes into four distinct clusters (Fig. 2). Cluster I comprises of ICPA 2043, ICPA 2047-4 and ICPA 2048, cluster II with genotypes ICPA 2049, 2051, 2078 and 2092, cluster III having ICPA 2188, 2189, 2199 and PRG 158 and cluster IV having genotypes Maruthi, Asha, MRG 1004 and Laxmi (Table 4). To test the goodness of fit of a clustering to a set of SSR data, cophenetic correlation coefficient or cophenetic value was estimated using the COPH and MXCOMP options in NTSYS-pc program. The cophenetic value of 0.94 obtained using the SSR data indicated a very good fit. Principal Component Analysis revealed that PC1, PC2, PC3 and PC4 accounted for 49.6, 30.8, 14.6 and 4.7% of total variation. Two dimensional and 3-dimensional plots were prepared by using all the four principal components. The 2-D plot differentiated all the fifteen genotypes into four different clusters which are in accordance with the UPGMA based clustering (Fig. 3). In the case of 3-D plot based on PCA, four distinct clusters have been identified with cluster I consisting of ICPA 2043, ICPA 2047-4 and ICPA 2048, cluster II having ICPA 2049, 2051 and 2078, Cluster III consisting of 2189, 2199 and PRG 158, cluster IV with genotypes ICPA 2092, ICPA 2188, Maruthi, Asha, MRG 1004 and Laxmi (Fig. 4). Genotypes ICPA 2092, ICPA 2188 have shown different clustering pattern in both the methods. In UPGMA based clustering ICPA 2092 is present in cluster II and ICPA 2188 in cluster III while in the case of PCA 3-D plot they have clustered in cluster IV. Except this discrepancy, both the UPGMA and PCA method have shown similar clustering of pigeonpea genotypes. The use of only few genotypes in pigeonpea breeding program for development of elite cultivars resulted in the

narrow genetic base among the released cultivars compared to wild relatives (Kumar *et al.*, 2004; Yang *et al.*, 2006). Similarly in our studies the released cultivars like Asha, Maruthi, Laxmi and MRG 1004 were grouped in a single cluster revealing the narrow genetic base of pigeonpea.

Progress through genetic improvement of yield potential has been limited, and the improved cultivars developed through breeding could not enhance the productivity of the crop in the last five decades. To break the yield barriers in pigeonpea one of the ways is to exploit the phenomenon of heterosis by hybrid breeding through cytoplasmic male sterile system. The advent of molecular marker techniques provides a new way for heterosis prediction, which effectively improves the efficiency of hybrid breeding. High heterotic effects were obtained from hybrids of genetically diverse parental plants through analysis of RAPD markers (Liu & Wu, 1998). However, there has been no report so far in using SSR markers to predict hybrid performance in legumes except mungbean (Sorajjapinun *et al.*, 2012).

The SSR primers used in the present study resulted in the amplification of 99 bands, with an average of 4.18. Based on the PIC primers PV20 and Ccac004 are the most informative having a value of 0.79. Cluster analysis has clearly classified the fifteen genotypes into four clusters with the popular varieties falling into a single cluster except PRG 158. Minimum value of similarity coefficients were observed between genotypes ICPA 2051 and MRG 1004 followed by ICPA 2049 and MRG 1004; ICPA 2078 and Laxmi indicating the existence of genetic diversity among the male sterile lines and popular cultivars and their possible utilization in hybrid breeding program. The heterotic patterns detected from various parental lines will be useful to the plant breeder to make cross combinations only from promising parents. This approach will help to reduce the field evaluation of large number of experimental hybrids.

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Table 1. Details of Polymorphic primers used in Pigeonpea genotypes

S. No.	Primer Code	Primer Sequence (5'-3')	Forward/ Reverse
1.	CCat011	TGCTCTAATGGCTAGTTCATCC AAACACTCATGGGTTAGATTCTCC	F R
2.	CCtc012	GAGGATTGCACCAAGCAACT GCACTGCTGGCCTTACCATA	F R
3.	CCtc 008	TCACAGAGGACCACACGAAG TGGACTAGACATTGCGTGAAG	F R
4.	CCtc003	ACACCACCATGCTAAAGAACAAG CCAAGCAAGACACGAGTAATCATA	F R
5.	CCtc006	GTAGAGGAGGTTCCAAATGACATA ATCTGTCTGGTGTTTTATGTGCT	F R
6.	PV20	GGCTCCACCATCGACTACTG GAATGAGGGCGCTAAGATC	F R
7.	PP5	GACAATTTGTCATGCATTGC TTGCAAAAACACTTGGTTGG	F R
8.	CCtc002	ACACCACCATGCTAAAGAACAAG CCAAGCAAGACACGAGTAATCATA	F R
9.	Ccac004	TCTTAGCATGTCCTCTATTTTCGT AGTACATTTCAAATCCACACATCC	F R
10.	CCtc007	TCACAGAGGACCACACGAAG TGGACTAGACATTGCGTGAAG	F R
11.	CCtc033	ATTCCTCTCTATCTCAGACTTTT TCGTGATGGAACAAGATACT	F R
12.	CCac036	ATCGGCTTTTGTCTTGATGA AAGCTACAAGGGATACACATGC	F R
13.	ICPM1D10	GGATTAACCAATTGTGAGTGAACC TGCACTTTATAAGCATTACCAACA	F R
14.	ICPM2B08	AGTTTGAAATTGCTTTTGGCT GAATTGGGAGAGACCGCATA	F R
15.	PB1	GGGCTTCCTTTTCTTCTC GTCTTCGTGTAAGTCATACT	F R
16.	PB12	GTCTTCGTGTAAGTCATACT CCGAGGTGCTCCAAGTGAC	F R
17.	PP4	GGAGCTATGTTGGAGGATGA CCTTTTGCATGGGTTGTAT	F R
18.	PP9	CACTTGGTTGGCTCAAGAAC GCCAATGAAATCACATCCTTC	F R
19.	PV14	CCCCACCAACTTTTCTTCC TAGAATTGACTTGGCGAGAA	F R
20.	CCtc004	ATCGCTTTGCATCCTTATC CTTCACGTACATTTTCGTTT	F R
21.	ICPM1E04	TTTTTATGGAATATTATGAGTTGGC AAGAGTTTCCCAACCCTGCT	F R
22.	PV13	ACCTGGTCCCTAAAACCAAT CAATGGAGCACCAAAGATCA	F R
23.	PV4	CTTCACCGATCTGACAGCAT TTTCTCCACTGGAACACTCG	F R
24.	CCac003	TGCTTCAAGTTGCTACCAG TCAAGGGAGGTGGACTACAAA	F R
25.	PV16	TGGTGAGAGAAGGACAATAGCA GCCGCTTGTGACGTTTATTT	F R
26.	PV23	CATCAACAAGGACAGCCTCA GCAGCTGGCGGGTAAAACAG	F R



Table 2. Allelic information and Polymorphic information content (PIC) obtained by using SSR markers in Pigeonpea genotypes

SSR locus	Alleles detected	No. of genotypes sharing an allele	No. of genotypes analyzed	Frequency of alleles	PIC
CCat011	a1	3	15	0.2	0.59
	a2	4	15	0.26	
	a3	8	15	0.53	
	a4	2	15	0.13	
CCtc012	a1	7	15	0.46	0.0
	a2	8	15	0.53	
	a3	7	15	0.46	
	a4	8	15	0.53	
CCttc008	a1	11	15	0.73	0.41
	a2	2	15	0.13	
	a3	9	15	0.6	
	a4	6	15	0.4	
CCttc003	a1	4	15	0.26	0.66
	a2	1	15	0.06	
	a3	5	15	0.33	
	a4	5	15	0.33	
	a5	3	15	0.2	
CCttc006	a1	6	15	0.4	0.55
	a2	2	15	0.13	
	a3	6	15	0.4	
	a4	3	15	0.2	
	a5	4	15	0.26	
PV20	a1	6	15	0.4	0.79
	a2	1	15	0.06	
	a3	1	15	0.06	
	a4	3	15	0.2	
PP5	a1	2	15	0.13	0.72
	a2	6	15	0.4	
	a3	4	15	0.26	
	a4	2	15	0.13	
	a5	1	15	0.06	
CCttc002	a1	2	15	0.13	0.57
	a2	5	15	0.33	
	a3	8	15	0.53	
	a4	2	15	0.13	
Ccac004	a1	2	15	0.13	0.79
	a2	3	15	0.2	
	a3	1	15	0.06	
	a4	4	15	0.26	
	a5	4	15	0.26	
CCttc007	a1	3	15	0.2	0.63
	a2	7	15	0.46	
	a3	5	15	0.33	
CCttc033	a1	5	15	0.33	0.56
	a2	4	15	0.26	
	a3	7	15	0.46	
	a4	3	15	0.2	
CCac036	a1	9	15	0.6	0.29
	a2	8	15	0.53	
	a3	3	15	0.2	
	a4	2	15	0.13	
	a5	1	15	0.06	
ICPM1D10	a1	2	15	0.13	0.72
	a2	6	15	0.4	
	a3	4	15	0.26	
	a4	1	15	0.06	
	a5	2	15	0.13	

Contd.,



Table 2. Contd.,

SSR locus	Alleles detected	No. of genotypes sharing an allele	No. of genotypes analyzed	Frequency of alleles	PIC
ICPM2B08	a1	6	15	0.4	0.48
	a2	9	15	0.6	
PB1	a1	4	15	0.26	0.78
	a2	4	15	0.26	
	a3	3	15	0.2	
	a4	2	15	0.13	
	a5	2	15	0.13	
PB12	a1	4	15	0.26	0.65
	a2	5	15	0.33	
	a3	3	15	0.2	
	a4	5	15	0.33	
	a5	2	15	0.13	
PP4	a1	4	15	0.26	0.16
	a2	5	15	0.33	
	a3	7	15	0.46	
	a4	10	15	0.66	
PP9	a1	4	15	0.27	0.49
	a2	1	15	0.07	
	a3	5	15	0.34	
	a4	1	15	0.07	
	a5	8	15	0.54	
PV14	a1	1	15	0.07	0.4
	a2	3	15	0.20	
	a3	11	15	0.74	
CCttc004	a1	7	15	0.46	0.63
	a2	5	15	0.33	
	a3	3	15	0.2	
ICPM1E04	a1	1	15	0.06	0.71
	a2	7	15	0.46	
	a3	2	15	0.13	
	a4	1	15	0.06	
	a5	3	15	0.2	
PV13	a1	5	15	0.33	0.67
	a2	7	15	0.46	
PV4	a1	5	15	0.33	0.78
	a2	5	15	0.33	
CCac003	a1	8	15	0.53	0.59
	a2	5	15	0.33	
	a3	2	15	0.13	
PV16	a1	8	15	0.53	0.66
	a2	3	15	0.2	
	a3	2	15	0.13	
	a4	1	15	0.06	
PV23	a1	2	15	0.13	0.71
	a2	6	15	0.4	
	a3	5	15	0.33	



Table 3. Jaccard's similarity coefficients based on SSR markers in fifteen pigeonpea genotypes

	ICPA 2043	ICPA 2047-4	ICPA 2048	ICPA 2049	ICPA 2051	ICPA 2078	ICPA 2092	ICPA 2188	ICPA 2199	ICPA 158	PRG	MARUTHI	ASHA	MRG 1004	LAXMI	
1	1.00															
2	0.47	1.00														
3	0.41	0.69	1.00													
4	0.25	0.30	0.39	1.00												
5	0.19	0.15	0.15	0.48	1.00											
6	0.12	0.14	0.18	0.44	0.52	1.00										
7	0.12	0.16	0.17	0.32	0.42	0.51	1.00									
8	0.08	0.12	0.10	0.20	0.31	0.41	0.44	1.00								
9	0.06	0.14	0.14	0.21	0.25	0.28	0.36	0.45	1.00							
10	0.08	0.18	0.16	0.24	0.23	0.28	0.33	0.36	0.73	1.00						
11	0.19	0.21	0.17	0.12	0.19	0.16	0.31	0.37	0.45	0.52	1.00					
12	0.18	0.20	0.21	0.13	0.09	0.15	0.25	0.23	0.15	0.22	0.36	1.00				
13	0.16	0.14	0.16	0.13	0.15	0.13	0.21	0.21	0.18	0.18	0.31	0.51	1.00			
14	0.12	0.12	0.15	0.08	0.06	0.10	0.15	0.17	0.21	0.21	0.27	0.34	0.47	1.00		
15	0.16	0.12	0.12	0.04	0.09	0.08	0.14	0.21	0.25	0.30	0.34	0.32	0.39	0.63	1.00	

Table 4. Cluster analysis in pigeonpea genotypes based on SSR data

Cluster	Genotypes
Cluster I	ICPA 2043, ICPA 2047-4 and ICPA 2048
Cluster II	ICPA 2049, 2051, 2078 and 2092
Cluster III	ICPA 2188, 2189, 2199, PRG 158
Cluster IV	Maruthi, Asha, MRG 1004 and Laxmi

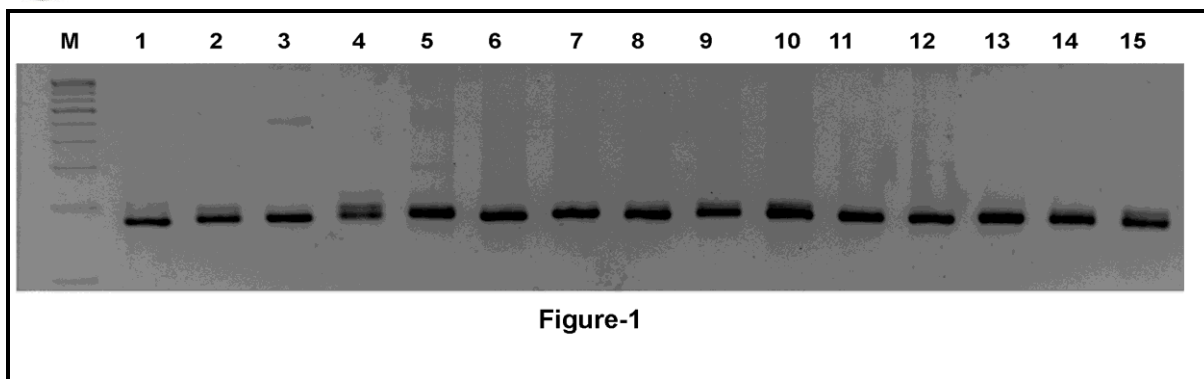


Fig. 1. SSR profile of pigeonpea genotypes using CCac036 marker

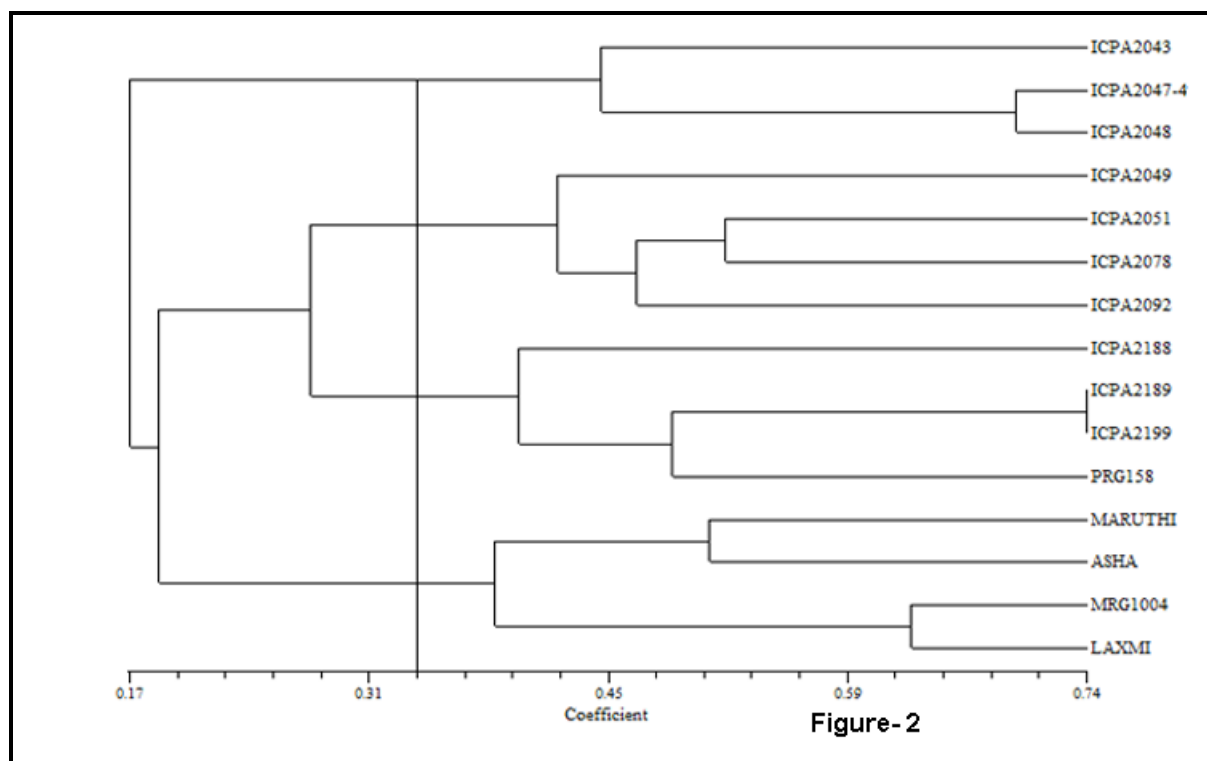


Fig. 2. Dendrogram of pigeonpea genotypes based on Jaccard's similarity coefficient of 25 polymorphic SSR loci

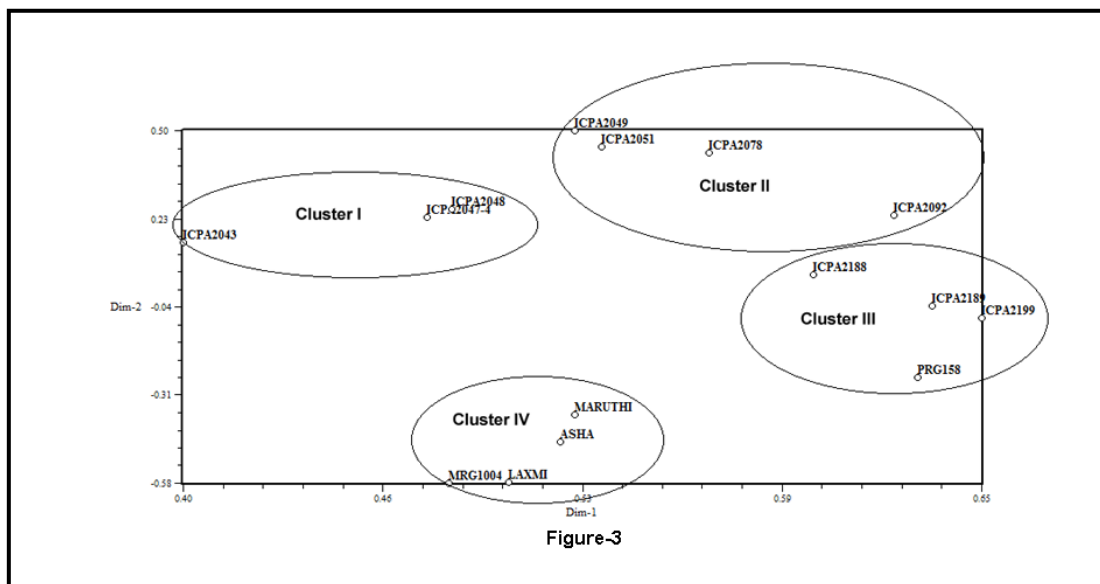


Fig. 3. Two dimensional plot of principal components 1, 2, 3 and 4 based on SSR data

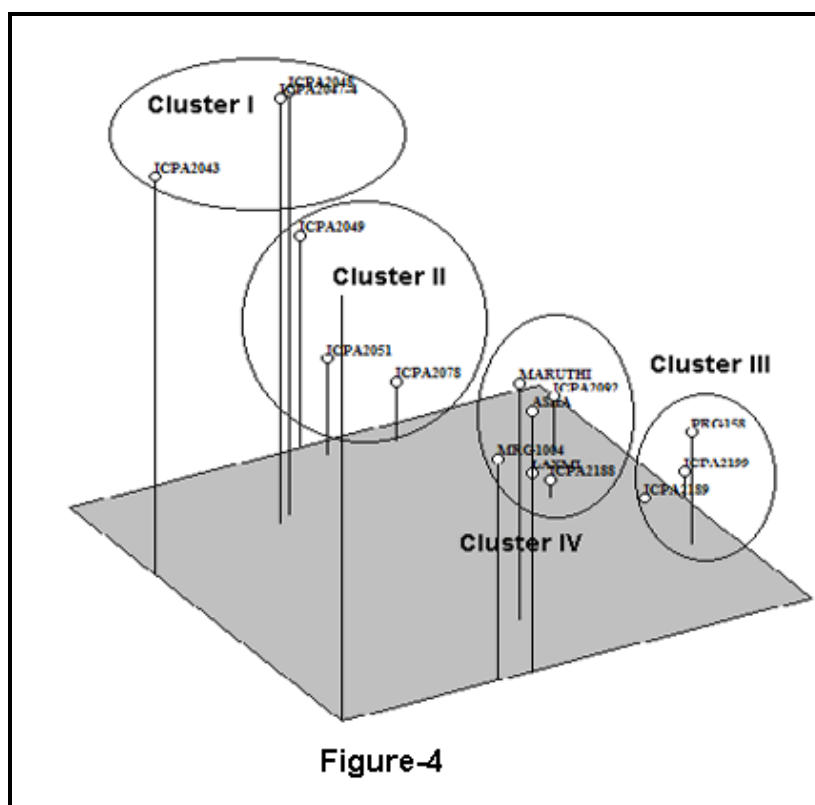


Fig. 4. Three dimensional plot of principal components 1, 2, 3 and 4 based on SSR data