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Molecular Diversity Analysis of Cowpea (*Vigna unguiculata* L.) Genotypes Determined by ISSR and RAPD Markers

Tushar J. Anatala, H. P. Gajera*, Disha D. Savaliya, Rinkal K. Domadiya, S. V. Patel and B. A. Golakiya

Department of Biotechnology, College of Agriculture, Junagadh Agricultural University, Junagadh- 362001, Gujarat, INDIA

Email: harsukhgajera@yahoo.com

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Abstract

The present study was carried out for the comparison of ISSR and RAPD markers for polymorphism pattern and molecular diversity analysis among ten cowpea (*Vigna unguiculata* L.) genotypes. Out of 50 ISSR primers, 18 primers produced total 103 bands across ten cowpea genotypes, of which 49 bands were polymorphic. A maximum polymorphism (85.70%) was obtained by UBC 815. Average numbers of polymorphic bands per UBC primer was 2.72. The UBC 834 was the best primer resulting good amplification with maximum PIC value (0.890). However, out of 50 RAPD primers, 14 RAPD primers produced 120 bands across ten genotypes, of which 81 bands were polymorphic. A maximum polymorphism (90.0%) was obtained with OPV-16 primer. Average number of polymorphic bands per RAPD primer was 5.78. The primer OPD-08 was the best primer resulting good amplification with maximum PIC value (0.923). Jaccard's similarity coefficient ranged from 68.40% to 92.90% for ISSR, for RAPD are 57.10% to 81.00% and for ISSR-RAPD coefficient data ranged from 85.70% to 63.50%.

Highlight

Cowpea genotype JCPL-2 was found most diverse based on ISSR and RAPD data. Both markers produced specific DNA finger prints for identification of cowpea genotypes.

Keywords: Cowpea, genetic diversity, ISSR, RAPD

Cowpea (*Vigna unguiculata* L.) is one of the oldest sources of human food and belonging to the family fabaceae. It is a nutritious crop for the human diet and cheaper livestock feed. It is used as a feed, fodder or pods as vegetable. Green tender pods form an excellent nutritious vegetable and have got a potential to solve the protein deficiency of human diet (Singh 1983). Tender marketable pod contained 85.3 % moisture, 35 % protein, 0.2 % fat, 2.0 % fiber, 8.1 % carbohydrates, 0.09 % mineral elements, 0.9 % niacin, 14.0 mg vitamins such as A, B etc. per 100 g of edible matter and 3.8 % iron (Swaminathan 1995) as well as good amount of calcium (Yadav and Bhutani 1988). Cowpea has a diploid chromosome (2n=22) with genome size is 600 Mb (Mishra *et al.*, 1985).

Molecular markers have acted as versatile tools and have been effectively employed in diverse fields like taxonomy, physiology, embryology and genetic engineering. Molecular

markers like RAPD and ISSR used in fingerprinting, population genetics and phylogenetic studies while SSR can be used for breeding, MAS, mapping (Nunome *et al.*, 2002). The RAPD and ISSR markers were used to study the DNA polymorphism in elite black gram genotypes (Souframanien and Gopalakrishna 2004). Among DNA based markers, arbitrary markers like ISSR and RAPD are useful for molecular characterization as these do not require prior information of the target genome. Therefore, attempts were made to study and compare ISSR and RAPD molecular marker techniques for varietal discrimination of cowpea. This investigation was initiated with the objective to assess and compare the efficiency of ISSR and RAPD markers in the assessment of genetic diversity among cowpea genotypes.

Materials and Methods

Plant material

Seeds of ten cowpea genotypes- advanced breeding lines (JCPL -2, JCPL-45, JCPL-48, JCPL-52, JCPL-97, JCPL-99, JCPL-116, JCPL-118, JCP-96-3-2-1 and JCP-96-24-2) were collected from Vegetable Research Station, Junagadh Agricultural University (JAU), Junagadh (Gujarat).

DNA isolation, PCR amplification and electrophoresis

Total genomic DNA was extracted from the leaves of 8 days seedlings by (CTAB) method (Doyle and Doyle 1987) with some modifications. In order to perform PCR based analysis, the DNA concentration was determined by Pico drop PET01 using software v2.08 (Picodrop Ltd., Cambridge U.K). The quantity was directly displayed as $ng.\mu l^{-1}$. The concentration of DNA was adjusted to 25 ng/µl for PCR reactions. Total 50 primers each for ISSR (UBC series) and RAPD operan series analysis were used. The 25 ng/µl reaction mixture contained 1x Taq buffer, 1.5 U Taq DNA polymerase, 200 µM dNTPs (Banglore Genei), 0.2 µM primers of ISSR and RAPD 50 ng template DNA. The PCR reaction was carried out in the Thermal Cycler (ABI, Applied bio-system) for amplification. The PCR amplification condition for ISSR, initial denaturation at 94°C for 5.0 min followed by 35 cycles of denaturation at 94 ºC for 1.0 min, primer annealing at Tm ±2 for 1.30 min and elongation at 72ºC for 2.0 min followed by final step of extension at 72 ºC for 5.0 min (Elham *et al.*, 2010). The PCR for RAPD amplification condition was initial denaturation at 94 ºC for 5.0 min followed by 35 cycles of denaturation at 94 ºC for 1.0 min, primer annealing at as per primer Tm ±2 for 1.30 min and elongation at 72ºC for 2.0 min followed by final step of extension at 72ºC for 7.0 min (Zannou *et al.*, 2008). The PCR products were mixed with 1µl of 6x DNA loading dye and separated on 1.2% agarose for ISSR and 1.2% agarose for RAPD which containing 4µg/µl ethidium bromide. After separations, gels were documented using gel doc systems (SYNGENE).

Statistical analysis

Clear and distinct bands amplified by ISSR and RAPD primers were scored for the presence (1) and absence (0) for the corresponding band among the genotypes. The binary data were subjected to UPGMA (Rohlf 1998) analysis using NTSYSpc version 2.02 (Anderson *et al.*, 1993).

Results and Discussion

ISSR analysis

Screening of 50 UBC primers was carried out using genomic DNA of two genotypes (JCPL-2, JCPL-45). Eighteen primers gave satisfactory clear banding pattern. The polymorphism patterns of 18 anchored ISSR primers across 10 cowpea genotypes were shown in (Table 1). The ISSR primers produced different numbers of DNA fragments, depending upon their inter simple sequence repeat motifs. The 18 ISSR primers produced total 103 bands across ten cowpea genotypes, of which 49 bands were polymorphic. The size varied from 264-2853 bp. Primer UBC 834 (Fig. 1A) and UBC 835 produced maximum 10 bands, while primer UBC 809, 810, 812, 814, 817, 820, 856 and 864 produced minimum 4 bands. A maximum 85.70% polymorphism was obtained with UBC 815. Average numbers of polymorphic bands per primer was 2.72. PIC values varied from 0.612 (UBC 856) to 0.890 (UBC 834) with an average of 0.775. Based on PIC value, the UBC 834 was the best primer resulting in good amplification with maximum PIC value (0.890).

Clustering pattern using ISSR data of ten cowpea genotypes is described in (Fig. 2A). Jaccard's similarity coefficient ranged from 68.40% to 92.90%. Dendrogram generated by ISSR molecular data gave two main clusters, clusters A and B. Cluster A included one genotype JCPL-2 and cluster B further divided in to B1 and B2. B1 sub-cluster further divided into B1 (a) and B1 (b). Sub-cluster B1 (a) included three genotypes JCPL-45, JCPL-48, JCPL-52 and Sub-cluster B1 (b) included five genotypes JCPL-97, JCPL-99, JCPL-116, JCPL-118 and JCP-96-24-2 while B2 sub-cluster included JCP-96-3-2-1. The results indicated that

Table 1: Number of amplified bands, percent polymorphism and PIC obtained by ISSR primers

Sr. No.	ISSR primer	Sequence 5'- 3'	Tm	TB	PB	% P	PIC value
1.	UBC 807	$(AG)_8T$	42.4	6	3	50.00	0.814
2.	UBC 808	$(AG)_8C$	46.7	9	2	22.20	0.888
3.	UBC 809	$(AG)_8G$	46.5	4	0	00.00	0.750
4.	UBC 810	$(GA)_8T$	42.8	4	2	50.00	0.735
5.	UBC 811	$(GA)_8C$	43.2	5	2	40.00	0.797
6.	UBC 812	$(GA)_8A$	44.3	4	3	75.00	0.717
7.	UBC 813	$(CT)_8T$	43.4	5	3	60.00	0.784
8.	UBC 814	$(CT)_8A$	41.3	4	2	50.00	0.666
9.	UBC 815	$(CT)_8G$	44.9	7	6	85.70	0.797
10.	UBC 816	$(CA)_8T$	51.1	8	6	75.00	0.849
11.	UBC 817	$(CA)_8A$	52.7	4	1	25.00	0.687
12.	UBC 820	$(GT)_8C$	50.3	4	2	50.00	0.735
13.	UBC 834	$(AG)_8YT$	45.3	10	4	40.00	0.890
14.	UBC 835	$(AG)_8YC$	45.5	10	4	40.00	0.885
15.	UBC 855	$(AC)_8YT$	51.9	6	3	50.00	0.807
16.	UBC 856	$(AC)_8YA$	49.8	4	2	50.00	0.612
17.	UBC 857	$(AC)_8YG$	53.7	5	3	60.00	0.790
18.	UBC 864	$(ATG)_6$	51.2	4	1	25.00	0.749
	Mean			5.72	2.72	47.10	0.775
	Total			103	49	-	-

TB: total number of bands, PB: polymorphic bands, %P: % polymorphism, PIC: polymorphic information content, Tm: annealing temperature

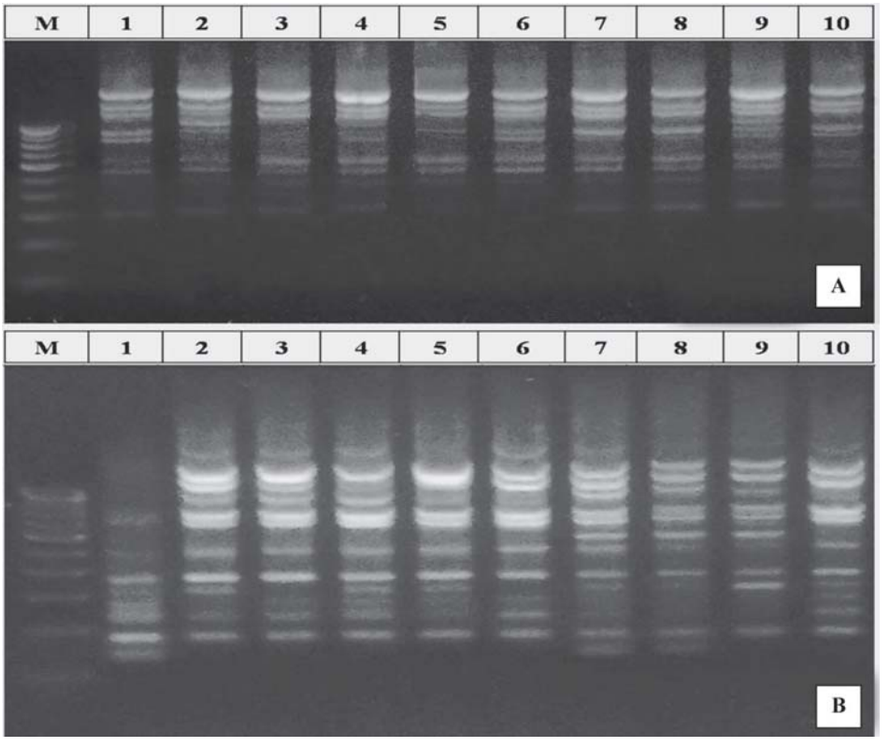

Fig. 1: PCR amplification profiles of 10 cowpea genotypes. (A) ISSR primer UBC-834. (B) RAPD primer OPD-08
(Lane M=100 bp DNA lader, lane 1 to 10 represents cowpea genotypes JCPL -2, JCPL-45, JCPL-48, JCPL-52, JCPL-97, JCPL-99, JCPL-116, JCPL-118, JCP-96-3-2-1 and JCP-96-24-2)

maximum similarity (92.90%) was found between JCPL-116 and JCPL-118. The minimum similarity (68.40%) was obtained between JCP-96-3-2-1 and JCPL-2 and it revealed that out of ten genotypes, JCPL-2 was highly variable.

Similar to present study, Singh *et al.,* (2010) used ISSR markers to study the DNA polymorphism in elite *Vigna* genotypes. Amplification of genomic DNA of the 20 *Vigna* genotypes using 12 ISSR primers yielded 94 fragments, of which 75 were polymorphic with an average of 6.25 fragments per primer. Number of amplified fragments ranged from four to ten and size of fragments varied from 100 to 1500 bp. Percentage polymorphism ranged from 55.55% (4824-039) to a maximum of 90% (4824-047), with an average of 79.5%. Cluster analysis grouped the 19 Mung bean (*V. radiata*) and one black gram (*V. Mungo*) genotypes into six main clusters. Cluster VI with 10 genotypes was the largest. Cluster I, II and V contained one genotype each. PM 06-34, the only genotype in cluster I, with a similarity coefficient value of 0.35, was the most divergent among the 20 genotypes. The Jaccard's similarity coefficient between different mung bean genotypes ranged from 0.263 to 0.85. Muthusamy *et al.,* (2008) studied genetic variation between 10 landraces of rice bean was evaluated using ISSR markers. The ISSR markers produced 479 amplification products, out of which 296 were polymorphic. The dendrograms constructed using RAPD and ISSR marker systems were highly correlated with each other as revealed by high Mantel correlation (r = 0.95). Jaccard similarity coefficient values ranged from 0.684 and 0.929 (ISSR), 0.571 to 0.810 (RAPD) and 0.635 to 0.857 (RAPD and ISSR) and mean similarity index value of 0.843, 0.750 and 0.793 for ISSR, RAPD and combined data, respectively. RAPD and ISSR marker systems were found to be useful for the genetic diversity studies in cowpea and identify variation within genotypes. Muthusamy *et al.,* (2008) also compared the clustering pattern of ISSR and RAPD markers in rice bean and found similar results.

RAPD analysis

Total 50 RAPD primers was used for amplification of cowpea genomic DNA. Among them 14 polymorphic primers gave satisfactory results which were used for further analysis of all cowpea genotypes. The banding pattern of 14 anchored RAPD primers using 10 cowpea genotypes were shown in (Table 2).

The RAPD primers produced different numbers of DNA fragments. The 14 RAPD primers produced 120 bands across ten genotypes, of which 81 bands were polymorphic. The size varied from 91-2282 bp. Primer OPD-08 (Fig.1B) produced maximum 15 bands, while primer OPB-19 produced minimum 4 bands. A maximum 90.00% polymorphism was obtained with OPV-16 primer. Average number of polymorphic bands per primer was 5.78. The PIC values varied from 0.682 (primer OPB-19) to 0.923 (primer OPD-08) with an average of 0.836. Based

Table 2: Number of amplified bands, percent polymorphism and PIC obtained by RAPD primers

Sr. No.	RAPD Primers	Sequence 5' – 3'	Tm	TB	PB	% P	PIC value
1.	OPA-12	TCGGCGATAG	36.7	8	7	87.50	0.813
2.	OPA-16	AGCCAGCGAA	40.5	7	6	85.70	0.827
3.	OPB-19	ACCCCCGAAG	41.9	4	3	75.00	0.682
4.	OPC-11	AAAGCTGCGG	40.5	8	7	87.50	0.794
5.	OPC-13	AAGCCTCGTC	32.6	10	6	60.00	0.878
6.	OPD-07	TTGGCACGGG	46.5	7	6	85.70	0.803
7.	OPD-08	GTGTGCCCCA	40.6	15	12	80.00	0.923
8.	OPF-09	CCAAGCTTCC	33.9	7	4	57.10	0.810
9.	OPJ-17	ACGCCAGTTC	33.7	9	6	66.70	0.857
10.	OPL-12	GGGCGGTACT	37.8	12	5	41.70	0.904
11.	OPO-04	AAGTCCGCTC	32.6	7	4	57.00	0.824
12.	OPP-13	GGAGTGCCTC	31.8	9	2	22.20	0.876
13.	OPP-19	GGGAAGGACA	32.7	7	4	57.00	0.825
14.	OPV-16	ACACCCCACA	33.8	10	9	90.00	0.895
	Mean			8.57	5.78	68.07	0.836
	Total			120	81	-	-

TB: total number of bands, PB: polymorphic bands, %P: % polymorphism, PIC: polymorphic information content, Tm: annealing temperature

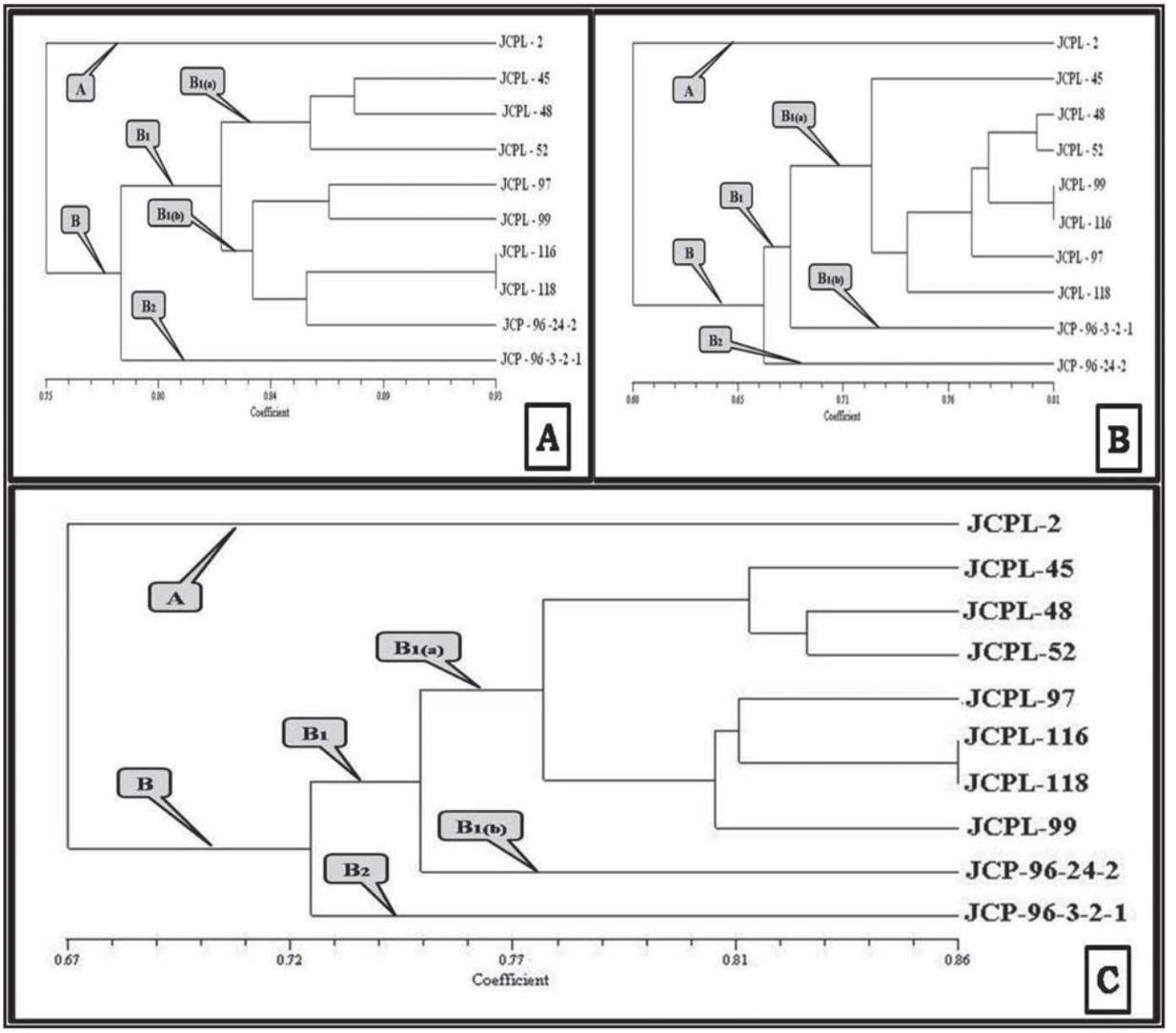

Fig. 2: Dendrogram derived from UPGMA cluster analysis using jaccard's similarity coefficient of (A) ISSR, (B) RAPD and (C) pooled of two markers

on PIC value, the primer OPD-08 was the best primer resulting in good amplification with maximum PIC value (0.923). Among them, there was maximum amplified allele size of 2282 bp (primer OPC-13) and minimum amplified allele size of 91 bp (primer OPC-11).

A dendrogram based on UPGMA analysis of ten cowpea genotypes with RAPD data is shown in (Figure 2B). Jaccard's similarity coefficient of RAPD ranged from 57.10% to 81.00%. Dendrogram generated by RAPD molecular data gave two main clusters, clusters A and B. Cluster A having only one genotype JCPL-2 minimum (60.00%) similarity with other 9 genotypes. Cluster B further divided into B1 and B2. B1 further divided into two sub clusters B1(a) and B1(b). B1(a) sub cluster included six genotypes like JCPL-45, 48, 52, 99, 116, 97 and 118. B1(b) Sub cluster comprised only one genotype JCP-96-3-2-1. Cluster B2 included only one genotype JCPL-96-24-2. So, the RAPD data revealed that JCPL-2 showed maximum variability compared to other nine genotypes. The results indicated that maximum similarity (81.00%) was found between JCPL-116 and JCPL-99.

The genetic diversity among ten Indian cultivars of cowpea was analyzed using 18 sets of RAPD markers by Malaviya *et al.*, (2012). A total of 181 bands with an average of 15 bands per primer were obtained. Out of 181 bands, 148 showed polymorphism (81.7%). The variation in genetic diversity among these cultivars ranged from 0.1742 to 0.4054. However, Motagi *et al.,* (2013) studied genetic diversity among 21 released varieties of cowpea using RAPD markers. A total of forty primers of two series (OPF01-20 and OPAG01-20) were used to screen the polymorphic primers and the profiles generated by such primers were used for analysis. The genetic similarity coefficients among genotypes varied between 0.44 and 0.82.

Malviya and Yadav (2010) reported the genetic diversity of 17 cultivars of pigeon pea using 17 RAPD markers. A total of 198 bands were scored corresponding to an average of 11.6 bands per primer with 148 bands showing polymorphism (74.7%). Nine out of eighteen primers gave more than 80% polymorphism. Jaccard's similarity coefficient ranged from 0.272 to 0.778. A dendrogram constructed based on the UPGMA clustering method revealed two major clusters. Cluster-I comprises of 12 cultivars which was further differentiated into two sub-clusters having six cultivars each. The cluster-II includes remaining five cultivars. The cultivar IPA-3088 was quite unique from the remaining cultivars as evident in the dendrogram. In present study RAPD finger printing detected more polymorphism loci (68.07%) than the ISSR finger printing (47.10%). The mean PIC value was 0.775 by ISSR and 0.836 for RAPD which suggested that both the marker systems were actually effective in determined polymorphism, Muthusamy *et al.,* (2008) also compared the ISSR and RAPD markers in rice bean and examined similar result.

Clustering pattern based on ISSR and RAPD combined data

The ISSR and RAPD data were combined for UPGMA cluster analysis. The UPGMA dendrogram obtained from the cluster analysis of ISSR and RAPD data is shown in Fig. 2C. The similarity coefficients ranged from 63.50% to 85.70%. Cluster analysis performed from the pooled data of markers generated a dendrogram that separated the genotypes into two distinct clusters, cluster A and B at 67.00% similarity. The first cluster A involved only one genotype JCPL-2 with highly variable. The cluster B further divided into sub cluster B1 and B2, sub cluster B1 further divided into B1(a) and B1(b). B1(a) involved, seven genotypes like JCPL-45, 48, 52, 97, 116, 118 and 99 while B1(b) comprised only one genotype JCP-96-24-2. Sub clusters B2 having one genotype JCP-96-3-2-1. Thus, combined ISSR and RAPD analysis revealed that out of ten genotypes JCPL-118 and JCPL-116 showed maximum similarity (85.70%). Minimum similarity between JCPL-2 and other 9 genotypes was found to be 63.50%. The dendrogram constructed using combined ISSR and RAPD data distinguished all genotypes by two clusters. Thus, it revealed that out of ten genotypes, JCPL-2 was highly variable. The dendrograms constructed using RAPD and ISSR marker systems were highly correlated with each other as revealed by high Mantel correlation (r = 0.95). Jaccard similarity coefficient values ranged from 0.684 and 0.929 (ISSR), 0.571 to 0.810 (RAPD) and 0.635 to 0.857 (RAPD and ISSR) and mean similarity index value of 0.843, 0.750 and 0.793 for ISSR, RAPD and combined data, respectively. RAPD and ISSR marker systems were found to be useful for the genetic diversity studies in cowpea and identify variation within genotypes. Muthusamy *et al.,* (2008) also compared the clustering pattern of ISSR and RAPD markers in rice bean and showed similar results.

Unique markers for DNA fingerprinting of genotypes

Presence or absence of amplified fragment by ISSR and RAPD markers were recorded as positive or negative markers. Total 6 positive and 6 negative ISSR markers were found to be unique to identify 7 cowpea genotypes (Table 3). However, total 18 varietal specific markers were formed by RAPD polymorphism out of which 8 were positive and 10 were negative unique fragments. Most diverse cowpea genotypes JCPL-2 were found dominant for producing both unique RAPD markers (positive and negative) to discriminate from other genotypes. Thus, ISSR and RAPD markers gave differentiated diversity to distinguished genotypes, individually with specific band.

From the above study, it can be concluded that molecular markers ISSR and RAPD are effective for determining polymorphism and very useful to study the diversity analysis. The clustering pattern of genotypes based on combined molecular data (ISSR and RAPD) showed similar pattern as shown in ISSR and RAPD clustering pattern individually that out grouped JCPL-2 genotype from other genotypes. Both the markers (ISSR and RAPD) produced unique positive and negative fragments as specific DNA finger print for identification of cowpea genotypes.

Table 3: Unique or rare loci produced by ISSR and RAPD Primers

Sr. No.	Genotypes	ISSR Primer	Marker size (bp)	RAPD Primer	Marker size (bp)
1.	JCPL – 2	UBC 855	+760	OPP-19	+824
		UBC 813	-1455	OPD-08	-792
				OPA-12	-1338
				OPJ-17	-1586
					-662
2.	JCPL – 45	…..	…..	OPA-12	+185
				OPF-09	+384
					+254
				OPC-13	-211
3.	JCPL – 48	UBC 834	-971	…..	…..
4.	JCPL – 52	UBC 856	+1396	…..	…..
5.	JCPL – 97	UBC 814	+321	OPJ-17	+404
		UBC 835	-494	OPO-04	-266
6.	JCPL – 99	UBC 835	+2800	…..	…..
		UBC 835	-978		
7.	JCPL – 116	…..	…..	…..	…..
8.	JCPL – 118	…..	…..	OPP-13	+814
9.	JCP-96-3-2-1	UBC 817	+528	OPL-12	-179
		UBC 864	-503		
10.	JCP-96-24-2	UBC 811	-1843	OPA-12	+1084
				OPJ-17	+1345
				OPV-16	-158
					-630
					-1633

(+): Positive Marker, (-): Negative Marker, (…..): Not detected

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