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Characterization and genetic diversity of cowpea (V*igna unguiculata* L.) genotypes linked to cowpea yellow mosaic virus

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ABSTRACT

The present research was carried out to investigate the genetic diversity among cowpea tolerant and susseptibe genotypes. For which the fifteen cowpea genotypes were manually infected with Cowpea yellow mosaic virus(CYMV) inoculum and to detect virus by a serological technique. The five most tolerant and sensitive each genotypes had been taken for genetic diversity study in which JCPL-2 and JCPL-11 was found most susceptible and tolerant genotype, having absorbance at 405nm 2.172 and 0.167 respectively. The RAPD, ISSR and SSR marker system were applied to the ten cowpea genotypes. For the RAPD data, based on PIC value, it can be said that primer OPAI-15 was the best primer resulting good amplification with maximum PIC value (0.764).Dendrogram constructed using the RAPD data clearly distinguished all genotypes. It revealed that JCPL-44 and JCPL-45 found in one cluster and shared maximum 96.00% similarity; however, genotype JCPL-2 have out grouped from other 9 genotypes and shared minimum 41.00% similarity. In case of ISSR data, based on PIC value it can be said that the primer 835 was the best primer resulting good amplification with maximum PIC value (0.837). Jaccard's similarity coefficient ranged from 0.625 and 0.906. The ISSR results indicated that maximum similarity 90.60% was found between JCPL-2 and JCPL-18, while minimum similarity 62.50% was obtained between JCPL-2 and JCP- 87. For the SSR data, based on PIC value it can be said that the primer VM-13 was the best primer resulting good amplification with maximum PIC value (0.668). Jaccard's similarity coefficient ranged from 0.500to 1.000. So SSR data revealed that JCPL-2 showed maximum variability compared to other nine genotypes. The combined RAPD, ISSR and SSR analysis revealed that out of ten genotypes JCPL-44 and JCPL-45 were showed maximum (91.0%) similarity. The lowest similarity of 58.30% was found between JCPL-2 and JCP-45.

Key words: Cowpea, CYMV, DAS ELISA, Genetic diversity, ISSR, RAPD, SSR.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, Leguminosae (2n = 2x = 22), a drought-tolerant crop, well-adapted to the drier regions of the tropics,where other food legumes do not perform well. The increasing agricultural production became an urgent issue since projections suggest that the global population will reach 9 billion people by the middle of this century (Godfray *et al.,* 2010). According to the estimation, 1 billion people will suffer from hunger because they do not have access to food in terms of protein deficit and micronutrient deficit. Cowpea yellow mosaic disease caused by cowpea yellow mosaic virus (CYMV) is one of the most serious diseases of cowpea. Indeed, CYMV causes the maximum damage to cowpea cultivars and it may cause yield reduction up to 80-100 % (Chant, 1959; and Williams, 1977). Cowpea yellow mosaic virus (Chant, 1959) is an RNA-containing virus with isometric particles about 28 nm in diameter. It has a limited host range, is transmitted mainly by beetles and readily by sap inoculation. To study the genetic diversity of cowpea genotypes associated with CYMV three different molecular markers viz. RAPD, ISSR and SSR were used. Simple Sequence Repeats (SSRs) are co-dominant markers that are routinely used to study genetic diversity in different crop species. These are DNA sequences that consist of two to five nucleotide core units such as (AT)n, (CTT)n and (ATGT)n,which are tandem repeats. The regions flanking the microsatellites are generally conserved among genotypes of the same species, allowing the selection of PCR primers that will amplify the intervening SSR in all genotypes. For identification of molecular markers linked to agronomically important genes, SSR is also one of the best choices as compared to RAPD and ISSR in a more polymorphic information or more cost effective manner, respectively.Therefore, this study was undertaken to investigate genetic relationship within and between yellow mosaic virus resistant and susceptible cowpea lines by using SSR, ISSR and RAPD markers.

MATERIALS AND METHODS

Plant materials and DNA isolation: Studies were undertaken to test the resistance or susceptible of cowpea

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germplasms against Cowpea yellow mosaic viral disease. Field experiments were conducted during 2010-2011 under irrigated conditions at Vegetable Research Station, JAU, Junagadh and in the Polyhouse at Department of Biotechnology J.A.U. Junagadh during 2011-2012. Fifteen cowpea genotypes were collected fromVegetable research station,Junagadh agricultural university Junagadh (Table 1) and Plants were infected with CYMVstrain of cowpea

Preparation of inoculum: Young leaves of 15-20 days old, showing characteristic mosaic symptoms were collected from infected cowpea plants, washed in tap water to remove the dust particles adhering to them and dried between folds of blotting paper. The leaves were then macerated in chilled mortar and pestle using potassium phosphate buffer (pH 7.0, 0.05M) at the rate of 1ml/gm of leaf tissue. The resultant pulp was squeezed through double layered muslin cloth and the extract thus obtained was used as standard inoculum.

Method of inoculation: To the standard extract, celite (600 mesh) was added at the rate of 0.025 g\ml of the extract. The inoculum was applied gently on the upper surface of the leaves with a small piece of absorbent cotton wool. Plants maintained to test mechanical inoculation and symptom expression and plants were kept under observation for 15-20 days in the pot.

Serological methods: Preparing for the test check all the components of ELISA Reagents. Prepare all buffer solutions according to the buffer formulations. Prepare a humid box for incubation steps and following the manufacturers' instructions and test results can be examined by eye, or measured on a ELISA plate reader at 405 nm. Development of yellow color in test wells indicates positive results. Wells in which there is no significant color development indicate negative results. Results may be interpreted after more than 60 minutes of incubation as long as negative control wells remain virtually clear.

DNA Isolation: Plants were raised in the polyhouse and young infected leaf tissues were used for DNA extraction. Young infected leaf tissues were harvested from the plants, washed to free from dirt and dust, and then quickly mopped, dried on blotting sheets. The leaves were dried and wrapped in tissue paper intern wrapped with blotting paper. These dry leaf tissues were used for DNA extraction Total genomic DNA was extracted from the leaves of young seedlings by Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987) with some modifications.The reagents and buffers for DNA isolation were prepared as per Oza *et al.* (2008) RAPD, ISSR and SSR primers, reactions and Agarose gel electrophoresis:

Various molecular marker techniques such as Randomoly Amplified Polymorphic DNA (RAPD), Inter SimpIe Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) were used for characterization of screened cowpea genotypes. Primers required for the above techniques were synthesized from Bangalore Genei, India, other chemicals for molecular studies were also received from Bangalore Genei. The basic reaction included25 ng template DNA, 0.2µM primer, 100 µM each of dATP,dGTP, dCTP and dTTP, 2 mM Mg2+ concentration and 0.5Uof *Taq*polymerase along with suitable 1× buffer (10 mMTris-Cl, pH 8.3, 50 mMKCl, 0.001% w/v gelatin) all taken in 25 µl final reaction volume. The reaction was carried out at94°C for 5 min as pre denaturation step, then the reaction was cycled 35 times at 94°C for 50 sec, 35°C for 1 min and extension at 72°C for 2 min. additionally a final cycle allowed extension for 5 min at 72°C in the Thermal Cycler(Bio-rad, USA). Care was taken to ensure that the set of genotypes to be compared were all analyzed in the same machine. The amplified products in all the above trials were Separated electrophoretic ally on 1.5% Agarose gels in 0.5×TBE buffer, visualized and photographed over a UV transilluminator after staining with ethidium bromide.

Table 1: Cowpea genotypes used in this study and their sorces

Sample no.	Genotype used in virus detection	Genotypes used for molecular diagnosis	Source
1	JCPL-2	JCPL-2	Vegitable research station JAU, junagadh
2	JCPL-11	JCPL-11	Vegitable research station JAU, junagadh
3	JCPL-14	JCPL-14	Vegitable research station JAU, junagadh
4	JCPL-15	JCPL-18	Vegitable research station JAU, junagadh
5	JCPL-18	JCPL-24	Vegitable research station JAU, junagadh
6	JCPL-23	JCPL-44	Vegitable research station JAU, junagadh
7	JCPL-24	JCPL-45	Vegitable research station JAU, junagadh
8	JCPL-28	JCPL-50	Vegitable research station JAU, junagadh
9	JCPL-44	JCPL-87	Vegitable research station JAU, junagadh
10	JCPL-45	JCP-96-25-1	Vegitable research station JAU, junagadh
11	JCPL-49	—	Vegitable research station JAU, junagadh
12	JCPL-50	—	Vegitable research station JAU, junagadh
13	JCPL-87	—	Vegitable research station JAU, junagadh
14	JCPL-104	—	Vegitable research station JAU, junagadh
15	JCP-96-25-1	—	Vegitable research station JAU, junagadh

Scoring and analysis of bands: Clear and distinct bands amplified by RAPD, ISSR and SSR primers were scored for the presence (1) and absence (0) for the corresponding band among the cultivars. The data were entered in to MS-Excel data sheet and subsequently analyzed using NTSYSpc version2.02 (Rohlf, 1998).

The data matrix was read by NTSYS-pc version 2.2 (Numerical Taxonomy and Multivariate Analysis System for Personal Computers, Exeter Software) developed by F.J. Rohlf and analyzed by the SIMQUAL program with Jaccard's similarity coefficient. SIMQUAL is a program for computing a variety of similarity and dissimilarity coefficients for qualitative data. The qualitative nature of the absence (0) or presence (1) state of a RAPD marker was used as the basis for similarity analysis among various cow pea genotypes. A matrix of 0 and 1 act as the input, and the output is a matrix of similarity or dissimilarity coefficients. The resultant similarity matrix was entered into SAHN clustering program, a tree matrix was produced and a dendrogram constructed using UPGMA. The assumption underlying the use of UPGMA clustering is the equal rate of evolution along all dendrogram branches. Dendrogram of publication quality were produced from the output tree file of SAHN by TREE (tree display) program in graphics mode. Clustering methods create clusters of the data, no matter whether there are true clusters in the data or not, so a check was made for the existence of true clusters. This was done by using the tree matrix produced by SAHN to calculate the cophenetic values of similarity or dissimilarity by the program COPH (cophenetic values). The cophenetic value matrix was compared with the original tree matrix for goodness of fit of the cluster analysis to the data. This type of cophenetic correlation was done by the MXCOMP (matrix comparison) program (Rohlf, 1998). The program MXCOMP plots the cophenetic value matrix against the original tree matrix, and computes the Cophenetic correlation coefficient (r) and the Mantel test statistic (Z).

The test criterion of Mantel test is as follows

n X_{ij} = off-diagonal elements of cophenetic value matrix

$Z = \Sigma X_{ij} Y_{ij} Y_{ij}$ = off-diagonal elements of original tree matrix

$i < j$ n = the number of elements of the matrices

As the cophenetic correlation coefficient is positively correlated to the Mantel test statistic and in standardized units, it is easier to use it as a measure of goodness of fit for a cluster analysis than the Mantel test statistic. The degree of fit can be referred as follows (Rohlf, 1998).

RESULTS AND DISCUSSION

The fifteen Cowpea genotypes were manually infected with CYMV inoculum by the standard procedure in polyhouse and detect virus by a serological technique in which five tolerant and susceptible each genotypes had been taken for the genetic diversity study . JCPL-2 and JCPL-11 were found to be most susceptible and tolerant having absorbance at 405nm was 2.172 and 0.167 respectively(Fig.1 and Table 2).The RAPD, ISSR and SSR data were combined for UPGMA cluster analysis. Similarity coefficient ranged from 58.30% to 91.04%. (Table 4 and Fig. 3). The amplification shown by different RAPD, ISSR and SSR primers (Fig. 2) and used primers series, sequences (5'-3'), Band size(bp), Total No. of Bands, No. of Polymorphic bands, Polymorphic % and PIC value shown in Table 3.The dendrogram constructed using combined RAPD, ISSR and SSR data distinguished all genotypes by two clusters. Thus, combined RAPD, ISSR and SSR analysis revealed that out of ten genotypes JCPL-44 and JCPL-45 were showed 91.04% maximum similarity(Figure 3).The lowest similarity of 58.30% was found between JCPL-2 and JCPl-45.

DAS-ELISA: For this purpose all the fifteen genotypes of cowpea used in the present investigation were mechanically inoculated with the cowpea viruses. A total of 15 plants were used for infection and monitored over three, four, five and six weeks after inoculation in which five susceptible and five tolerant cowpea genotypes were taken for further studies and the five moderate genotypes were omitted. The method of mechanical inoculation of seedlings in the polyhouse with CYMV inoculum at a standard level of 1:200 dilutions of freeze-dried, infected leaves followed by screening of the lines with the highest levels of tolerancy or susceptibility in field experiment through enzyme linked immunosorbent assay (ELISA). The fifteen cowpea genotypes JCPL-2, JCPL-11, JCPL-14, JCPL-15, JCPL-18, JCPL-23, JCPL-24, JCPL-28, JCPL-44, JCPL-45, JCPL-49, JCPL-50, JCPL-87, JCPL-104, JCP-96-25-1 were used for detection of virus in which the genotypes JCPL-2, JCPL-14, JCPL-24 JCPL-50,and JCP-96-25-1 having absorbance at 405nm is 2.17292, 1.12735, 0.800125, 0.700261, 1.15199 respectively and the genotypes JCPL-11, JCPL-18, JCPL-44, JCPL-45, JCPL-87 having absorbance at 405nm is 0.167178, 0.197752, 0.225636, 0.186842, 0.20629 respectively. The positive control having absorbance at 405nm is 6.00000 and negative control having absorbance at 405nm is 0.296385 (Fig. 1 and Table 2). According to this data concluded that JCPL-2, JCPL-14, JCPL-24 JCPL-50,and JCP-96-25-1 having virus so it is susceptible to CYMV and other five genotype like JCPL-11, JCPL-18, JCPL-44, JCPL-45, JCPL-87 have no virus so it was tolerant to CYMV and the next five genotype were omitted. This lack of repeatability and the difficulty of determining virus buildup using average ELISA values led us to use only the percentage of infected plants in determining possible tolerant lines in the later field testing. JCPL-2 had the maximum absorbance at 405nm (2.17292) and JCPL-11 had minimum absorbance at 405nm (0.167178). So JCPL-2 had maximum susceptibility and JCPL-11 had maximum tolerant cowpea genotypes. Salim Khan *et al* (2003) have worked for virus identification *in vitro* regenerated plantlets of six potato varieties such as, Cardinal,

Table2: DAS-ELISA reading of 15 cowpea genotype with positive and negative control

Well	Genotyes	Group	Abs.	Wavelength	Type
A01	Jcp-96-25-1	Assay	1.15199	405	Susceptible
B01	Jcpl-45	Assay	0.186842	405	Resistant
C01	Jcpl-15	Assay	0.61433	405	Moderate
D01	Jcpl-24	Assay	0.800125	405	Susceptible
E01	**Jcpl-11**	Assay	0.167178	405	Resistant
F01	Jcpl-23	Assay	0.647914	405	Moderate
G01	Jcpl-14	Assay	1.12735	405	Susceptible
H01	Jcpl-50	Assay	0.700261	405	Susceptible
H02	Positive control	Assay	6	405	Positivecontrol
G02	Jcpl-18	Assay	0.197752	405	Resistant
F02	Jcpl-87	Assay	0.20629	405	Resistant
E02	Jcpl-28	Assay	0.755003	405	Moderate
D02	Jcpl-44	Assay	0.225636	405	Resistant
C02	Jcpl-2	Assay	2.17292	405	Susceptible
B02	Jcpl-49	Assay	0.74468	405	Moderate
A02	Jcpl-104	Assay	0.539349	405	Moderate
A03	Negative control	Assay	0.296385	405	Negativecontrol

Table 3: Primers, Size, number of amplified bands, per cent polymorphism and PIC obtained by RAPD,ISSR and SSR primer respectively

RAPD Primers	Sequence (5'-3')	Allele/Band size (bp)	Total No. of Allele/ bands (A)	No. of Polymorphi c bands (B)	% Polymorphi sm (B/A)	PIC value
OPA-16	AGCCAGCGAA	237-1354	4	4	100	0.692
OPAI-15	GACACAGCCC	285-2346	5	2	40	0.764
OPK-18	CCTAGTCGAG	556-2653	3	3	100	0.615
OPO-11	GACAGGAGGT	233-508	4	4	100	0.749
ISSR PRIMERS						
822	$(TC)_8A$	322-911	6	6	100	0.778
834	$(AG)_8YT$	195-1395	5	4	80	0.787
835	$AG)_8YC$	230-1130	7	5	71.43	0.837
808	$(AG)_8C$	658-4479	5	3	60	0.789
SSR PRIMER						
VM13	CACCCGTGATTGCTTGTTG GTCCCCTCCCTCCCACTG	155-407	3	3	100	0.668

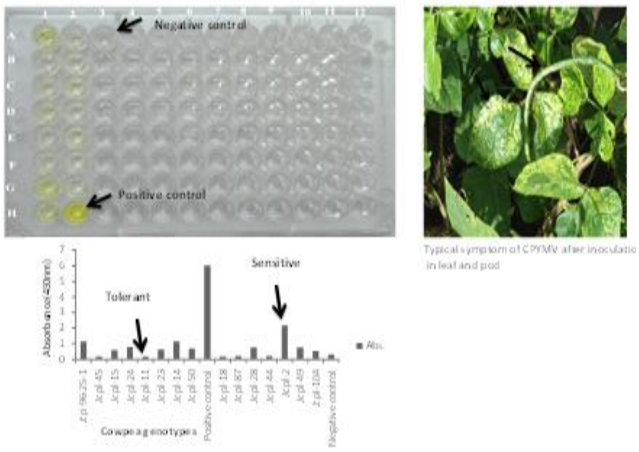

Fig 1: Serological study of the genotypes which was mechani cally infected in polyhouse and their typical symptom

Diamant, Dhera,Multa, Cilena and Sieglinde were mechanically inoculated with seven potatoviruses, namely A, Y, V, M, S, X and PLRV and identified through double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Ojuederie *et al* (2009) had study that was carried out to detect the presence of seed borne viruses in fourteen cowpea accessions screened from 300 regenerated cowpea germplasm from the gene bank collection of the International Institute of Tropical Agriculture (IITA) in Nigeria.

MOLECULAR MARKERS

Clustering pattern using RAPD data: Clustering pattern using RAPD data a dendrogram based on UPGMA analysis grouped the ten cowpea genotypes into two main clusters A, and B with Jaccard's similarity coefficient ranging from 41% to 96%. The dendrogram constructed using the RAPD data

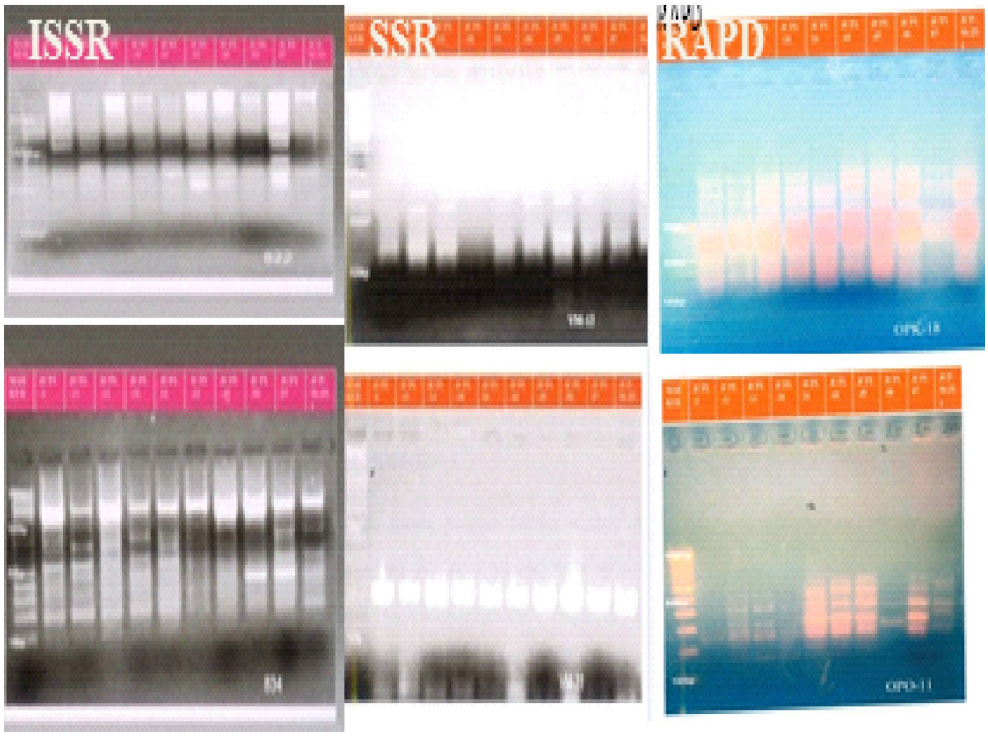

Fig 2: Typical ISSR, SSR and RAPD, profiles obtained for the 10 cowpea DNAs with primers respectively. Lane marked as marker in each gel panel was for the l DNA double digested with *E.coRI and hindIII* as fragment size marker

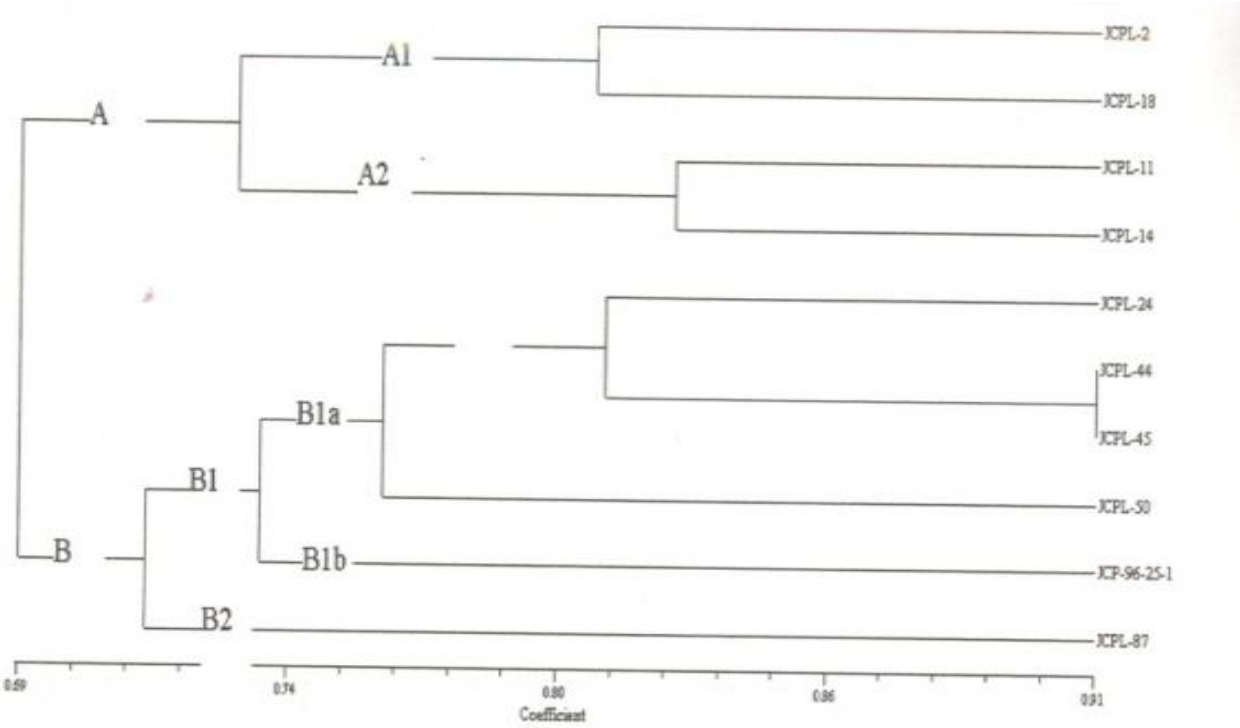

Fig 3: Dendrogram depicting the genetic relationship among 10 cowpea genotypes based on pooled data of RAPD, ISSR and SSR

Table 4: Jaccard's similarity coefficient of 10 Cowpea genotypes based on pooled data of RAPD,ISSR and SSR

	JCPL-1	JCPL-1	JCPL-1	JCPL-2	JCPL-4	JCPL-4	JCPL-5	JCPL-8	JCP-96-25-	
JCPL 2	1	4	8	4	4	5	0	7	1	
JCPL-2	1.000									
JCPL-11	0.738	1.000								
JCPL-14	0.691	0.823	1.000							
JCPL-18	0.807	0.778	0.727	1.000						
JCPL-24	0.632	0.714	0.694	0.692	1.000					
JCPL-44	0.616	0.716	0.743	0.671	0.785	1.000				
JCPL-45	0.538	0.732	0.736	0.686	0.833	0.910	1.000			
JCPL-50	0.676	0.815	0.69	0.77	0.731	0.757	0.803	1.000		
JCPL-87	0.560	0.680	0.684	0.633	0.675	0.770	0.763	0.694	1.000	
JCP-96-25-1	0.676	0.732	0.715	0.686	0.704	0.778	0.720	0.750	0.671	1.000

clearly distinguished all genotypes. Genotypes JCPL-44 and JCPL-45 were found in one cluster with maximum 96% similarity. RAPD data revealed that two genotypes JCPL-2 and JCPL-44 showed minimum similarity 41%. Earlier, low to moderate polymorphism was observed while analysing 32 Indian mung bean cultivars using 21 RAPD primers (Lakhanpaul *et al*. 2000).

Clustering pattern using ISSR data: A dendrogram based on UPGMA analysis of ten cowpea genotypes with ISSR data is shown in Jaccard's similarity coefficient ranged from 62.50% to 90.60%. Thus, the results indicated that maximum similarity was found 90.60% between JCPL-2 and JCPL- 18. When Singh *et al.* (2010) were used the 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. Percentage polymorphism ranged from 55.55% (4824-039) to a maximum of 90% (4824-047), with an average of 79.5%.The Jaccard similarity coefficient between different Mungbean genotypes ranged from 0.263 to 0.85.

Clustering pattern using SSR data: Thus, the results indicated that maximum similarity was found 100.0% among JCPL-11,JCPL-14, JCPL-44, JCPL-45, JCPL-50 and JCP-96-25-1. The amplification shown by different RAPD, ISSR and SSR primers (Fig. 2) and used primers series, sequences (5'-3'), Band size(bp), Total No. of Bands, No. of Polymorphic bands, Polymorphic % and PIC value shown in Table 3.

Clustering pattern based on RAPD, ISSR and SSR data: The RAPD, ISSR and SSR data were combined for UPGMA cluster analysis. The UPGMA dendrogram obtained from the cluster analysis of RAPD, ISSR and SSR. Similarity coefficient ranged from 58.30% to 91.04%. Cluster analysis performed from combining data of markers generated a dendrogram that separated the genotypes into two distinct clusters, cluster A and B. The first cluster A involved sub cluster A1and A2. A1 involves two genotypes JCPL-2 and JCPL-18 and A2 involves JCPL-11 and JCPL-14. 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, four genotypes like JCPL-24, JCPL-44, and JCPL-45 and JCPL-50 and while B1(b) comprised JCPL-96-25-1, While the sub clusters B2 comprised JCP L-87 genotype. The dendrogram constructed using combined RAPD, ISSR and SSR data distinguished all genotypes by two clusters. Thus, combined RAPD, ISSR and SSR analysis revealed that out of ten genotypes JCPL-44 and JCPL-45 were showed 91.04% maximum similarity (Fig. 3).The lowest similarity of 58.30% was found between JCPL-2 aJCPl-45. Same result of highest variability between these genotypes was found by ISSR markers individually. Dendrogram depicting the genetic relationship among 10 cowpea genotypes based on pooled data of RAPD, ISSR and SSR. Gioi *et al*. (2010) used microsatellite markers to investigate the genetic basis of cowpea yellow mosaic virus (CYMV) resistance in 40 cowpea lines. The polymorphism information content (PIC) of these SSR markers ranged from 0.30 to 0.72(Table 3) The present study has also shown the usefulness of the method not only to assess the range of diversity in the germplasm but also to enable potential clustering of accessions on basis of their affinities to each other in agronomic performance traits and JCPL11 may be use as a donar for Marker assisted backcrossing for the development of the new CYMV tolerant variety and also a source of new QTL/gene identification for high yield and other trait.

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