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DNA Fingerprinting and Genetic Diversity Analysis Using RAPD, SSR And ISSR Markers In Mungbean (*Vigna radiata* (L.) Wilczek)

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Abstract: *Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) and Inter Specific Sequence Repeats (ISSR) markers were used to identify the genotypes through DNA fingerprinting and to study the DNA polymorphism in twelve mungbean genotypes. A total of thirty primers were used in this study and eighteen of them generated polymorphic pattern. Amplification of genomic DNA of these primers yielded 159 fragments out of them 117 are polymorphic and showed 73% polymorphism. Number of polymorphic primers obtained with these primers ranged from two fragments (VR062) to seventeen fragments (OPB12). Percentage of polymorphism ranged from 50% (OPB1) to 100% (OPD6 and VR0155). The Jaccard`s similarity indices based on RAPD, SSR and ISSR primers were subjected to UPGMA cluster analysis and twelve genotypes were grouped into five clusters. Based on the similarity coefficients, the cross combinations IPM-02-19 X AKM-9904, LGG 450 X WGG 2 and MGG 347 X VG-6197A could be suggested for initiating breeding programme to develop high yielding mungbean genotypes. Similarly the fingerprinting pattern with markers VR0155, OPB12, OPD7, OPD11 and ISSR842 for MGG 347, OPB12 for LGG 450, OPD7 for AKM 9904, OPD11 for EC 396117 and ISSR842 for PUSA 9531 could be utilized for identification of these genotypes.*

Keywords: *DNA fingerprinting, Genetic Diversity, Mungbean, RAPD, SSR, ISSR*

I. Introduction

Mungbean (*Vigna radiata* (L.) Wilczek; 2n=22) is an important food legume and protein supplement in Indian vegetarian diet. It is widely cultivated in India and occupy third place after pigeon pea and chickpea. India is the world's largest producer, consumer and importer of pulses as they are the major protein source in the largely vegetarian Indian diet. However, the per capita availability of pulses has declined from 60.7 g day^{-1} in 1951 to 41.9 g day^{-1} in 2013 as against the FAO/WHO's recommendation of 80 g day^{-1} (Economic survey 2014-15). The perusal of this statistics clearly shows that the production, productivity and consumption levels are less. Hence, there is an immediate need to develop cultivars with increased productivity. Many breeding programmes have been initiated to develop high yielding varieties to various niches. However, the progress is not significant since mungbean is a highly self- pollinated crop and lacks diverse gene pools. In order to enhance genetic potential, there must be a comprehensive understanding of the amount and pattern of genetic diversity that exists within and between the available cultivars. The genetic knowledge regarding the variation available in the germplasm helps to compare each germplasm and to choose diverse parents for hybridization.

Though, genetic diversity assessment has been made traditionally through morphological characters that are often limited in number, have complex inheritance and vulnerable to environmental conditions, of late, genetic diversity analysis based on DNA based markers are being used, since these markers are available in abundance and clearly allow the comparison of genetic material at juvenile phase avoiding any environmental influence on gene expression. It is also well documented that the DNA markers have many advantages over the traditional morphological markers, however, genetic diversity studies in mungbean using diverse DNA markers are limited in number and needs immediate attention to exploit this crop by thorough understanding at molecular level. Despite, Random Amplified Polymorphic DNA (RAPD) markers offer simple, efficient and economic method for diversity analysis and cultivar identification, Simple Sequence Repeats (SSRs) and Inter Specific Sequence Repeats (ISSRs) show a large and stable polymorphism due to variation in the number of repeat units and currently the most preferred molecular marker system owing to their highly desirable properties viz., abundance, hyper variability and suitability for high throughput analysis. Hence, these three different types of primers were used in the present investigation to study the DNA fingerprinting and diversity at molecular level.

II. Materials And Methods

2.1 Plant material and DNA extraction

Seed material of twelve mungbean genotypes (AKM 9904, EC396117, IPM-02-19, KM-8-657, LGG 450, MGG 347, MGG 350, MH 565, ML 145, PUSA 9531, VG 6097A and WGG 2) were collected and grown under laboratory condition at department of Genetics and Plant Breeding, S. V. Agricultural College, Tirupati. Total genomic DNA was isolated from the young leaves following the CTAB method described by Murray and Thompson (1980) with minor modifications. The quality was estimated by measuring O.D. at 260/280 nm and 260 nm respectively in a Nanodrop Spectrophotometer. Purity of genomic DNA was also checked on 0.8 per cent agarose gel before proceeding further for PCR analysis.

2.2 PCR amplification and electrophoresis

2.2.1 Random Amplified Polymorphic DNA (RAPD)

RAPD amplifications were carried out in a 25µl reaction mixture containing 2.5µl of 10X Taq buffer, 1.0 µl of 10 mM dNTPs, 2.0 µl of primer (20 picomoles/µl), 0.3 µl of Taq polymerase enzyme (conc. 1.5. U/µl), 1.2 µl of Di methyl Sulphoxide (DMSO), 3.0 µl of template DNA (15 ng/µl) and 13 µl of Milli-Q-water. Amplification conditions were followed with initial denaturation at 94˚C for 2 min and 40 cycles of amplification at 94˚C for 1 min, 36˚C for 1 min, 72˚ for 2 min followed by 7 min at 72˚C. Amplified products were separated on 1 per cent agarose in 1X TBE buffer at 100 volts. The gel was stained with Ethidium Bromide (4µl/ 100ml of gel) and visualized by illumination under UV light in gel documentation system. The size of the amplification products was determined by comparison with 1kb ladder.

2.2.2 Simple Sequence Repeats (SSR)

SSR amplifications were carried in a 10 µl reaction mixture containing 1 µl of 10X Taq buffer, 1.0 µl of 10 mM dNTPs, 0.4 µl of 25 mM $Mgcl_2$, 1.0 µl of Forward primer (20 picomoles/ µl), 1.0 µl Reverse primer (20 picomoles/ µl), 0.03 µl Taq polymerase enzyme (conc. 1.5 U/µl), 2.0 µl of template DNA (15ng/ µl) and 3.6 µl of Milli-Q-water. Amplification conditions were followed with initial denaturation of 94˚C for 4 min and 35 cycles of amplification at 94˚C for 45 sec, primer specific temperature for 1 min, 72˚C for 1.30 min followed by 7 min at 72˚C. Amplified products were separated on 9% Polyacrylamide Gel Electrophoresis (PAGE) at 100 volts. The gel was stained with Ethidium Bromide (4µl/ 100ml of gel) and visualized by illumination under UV light in gel documentation system. The size of the amplification products was determined by comparison with 50kb ladder.

2.2.3 Inter Specific Sequence Repeats (ISSR)

ISSR amplifications were carried in a 25 µl reaction mixture containing 2.5 µl of 10X Taq buffer, 1.0 µl of 10mM dNTPs, 2.5 µl of 25 mM $Mgcl_2$, 3.0 µl of Taq polymerase enzyme (conc. 1.5U/µl), 3.0 µl of template DNA (15ng/ µl) and 13µl of Milli-Q-water. Amplification conditions were followed with initial denaturation at 94˚C for 4 min and 35 cycles of amplification at 94˚C for 30 sec, primer specific temperature for 45 sec and 72˚C for 2 min followed by 7 min at 72˚C. Amplified products were separated on 2 per cent agarose in 1X TBE buffer at 100 volts. The gel was stained with Ethidium Bromide (4 µl/ 100 ml of gel) and visualized by illumination under UV light in gel documentation system. The sizes of the amplification products were determined by comparison with 1 kb ladder.

The details of RAPD, SSR and ISSR primers used, their sequences and specific temperature for each primer are given in Table 1.

2.3 Data analysis

The amplified products for RAPD, SSR and ISSR analysis were scored visually based on the presence (taken as "1") or absence (taken as "0") of band for each primer. Each fragment was treated as a unit and only clear and unambiguous bands were scored. The data were used to generate Jaccard`s coefficients for expressed RAPD, SSR and ISSR bands. The Jaccard`s coefficient were used to construct a dendrogram using the Unweighted Pair Group Method with Arithmetic averages (UPGMA). Statistical Package for Social Sciences (SPSS) package was used for the cluster analysis.

III. Results And Discussion

Analysis of molecular diversity among twelve mungbean genotypes with eighteen (ten RAPDs, five SSRs and three ISSRs) out of thirty five primers gave scorable, clear and consistent amplification profiles with all the twelve mungbean genotypes. All these eighteen primers produced a total of 159 fragments, out of them 117 are polymorphic and showed 73% polymorphism. All the eighteen primers along with their total number of bands, total number of polymorphic bands and percentage polymorphism were presented in Table 2. Among

these, RAPD primers generated a total of 94 fragments of which 70 were polymorphic and showed 74% polymorphism. Similarly, the SSR primers generated a total of 30 fragments of which 21 were polymorphic and showed 70% polymorphism and finally ISSR primers generated a total of 35 fragments out of them 26 were polymorphic and showed 74% polymorphism. Out of eighteen primers, two primers (VR0155 and OPD6) showed 100% polymorphism and rest of them showed polymorphism ranging from 50-85%. Karuppanapandian et al. (2006) and Sonia et al. (2012) also reported high amount of polymorphism using RAPD markers in mungbean. Gupta et al. (2013) also reported similar type of higher polymorphism using SSR markers in black gram. Palaniappan and Murugaiah (2012) also reported higher amount of polymorphism using SSR markers. Chattopadhyay et al. (2005) reported higher amount and also higher efficiency of polymorphism using ISSR markers.

Jaccard`s similarity coefficient were calculated to establish the genetic relationship among twelve genotypes using eighteen polymorphic primers and presented in Table 3. Jaccard`s genetic similarity values ranged from 0.169 to 0.663, indicating the presence of wide range of genetic diversity at molecular level among the twelve genotypes. The similarity coefficient value was highest (0.663) between the genotypes EC-396117 and IPM-002-19, indicating that these genotypes showed maximum degree of similarity in their genetic makeup. However, the minimum value of similarity coefficient was observed between genotypes MGG 347 and WGG 2 (0.169) and followed by genotypes WGG 2 and KM-8-657 (0.177) indicating that, these genotypes were highly diverse pairs.

The Jaccard`s similarity coefficient values were used to construct the UPGMA (Unweighted Pair Group Method on Arithmetic Average) dendrogram showed in Fig 1. Cluster analysis grouped the 12 mungbean genotypes into five clusters (Table. 3) of which cluster I and cluster V had four genotypes each and cluster IV had two genotypes. EC 396117, IPM-02-19, LGG 450 and KM-8-657 of cluster I were closely related to each other indicating the presence of low genetic variation between them. Similarly, PUSA 9531, VG-6197A, MGG 350 and WGG 2 of cluster V and MH 565 and ML 145 of cluster IV were also found to be closely related.

DNA fingerprinting makes it possible for researchers to pinpoint specific fingerprint and accurately identify a particular genotype. It has been found that DNA fingerprinting profile for the genotype MGG 347 by primers, VR0155 in 150bp (Fig 2), OPB12 in 600bp and 250bp (Fig 4), OPD7 in 1100bp, OPD11 in 750bp and ISSR842 in 500bp (Fig 3) positions were unique. Similarly, the genotypes LGG 450 with primer OPB12 in 890bp (Fig 4), AKM 9904 with primer OPD7 in 1480bp, EC 396117 with primer OPD11 in 700bp and PUSA 9531 with primer ISSR842 in 840bp (Fig 3) showed unique bands. Hence, the fingerprint profiles of these genotypes could be exploited for future varietal identification.

In present study, attempt has been made to combine RAPD, SSR and ISSR markers for diversity analysis in selected lines of mungbean. Thus from the above investigation, it may be concluded that molecular analysis revealed substantial polymorphism in mungbean. The technique may be used to obtain reasonably precise information on genetic relationship among mungbean genotypes. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically in the breeder's working collection of mungbean. Different methodological approaches such as morphological, Isoenzyme, protein and DNA markers have employed in the course of years to evaluate the genetic diversity in crop plants. Among them, DNA based marker approach has been found to be superior, because of its capability to reveal more polymorphism (Mignouna et al., 1998). Extensive molecular diversity analysis was done by using different molecular markers viz., Sonia et al. (2012) using RAPD markers, Sunitha et al. (2012) using 24 ISSR markers and Wang et al. (2012) using 15 SSR markers reported polymorphism in different mungbean genotypes used in their study. Genetic diversity analysis using PCR based markers (RAPD, SSR and ISSR) revealed that, higher marker indices were obtained for ISSR markers, which proved to be the most efficient marker system in terms of average heterozygosity values (Akanksha et al. (2014)).

IV. Conclusion

In conclusion, considering the diversity analysis, crossing between IPM-02-19 (cluster I) X AKM 9904 (cluster II), LGG 450 (cluster I) X WGG 2 (cluster IV) and MGG 347 (cluster III) X VG- 6197A (cluster V) could be suggested for initiating breeding programme to develop high yielding varieties, since these genotypes not only showed high molecular diversity but also showed proven yield performance in various yield trails. Similarly, the fingerprinting pattern with markers VR0155, OPB12, OPD7, OPD11 and ISSR842 for MGG 347, OPB12 for LGG 450, OPD7 for AKM 9904, OPD11 for EC 396117 and ISSR842 for PUSA 9531 could be utilized for identification of these genotypes.

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Table 1. List of RAPD, SSR primers and ISSR primers used, their sequence and annealing temperature

S. No	Primer	Sequence (5`-3`)	Annealing temparature(°c)
1	OPA-01	CAGGCCCTTC	36
2	OPB-01	CTTTCGCTCC	36
3	OPB-02	TGATCCCTGG	36
4	OPB-03	CATCCCCCTG	36
5	OPB-06	TGCTCTGCCC	36
6	OPB -12	CCTTGACGCA	36
7	OPC-01	TTCGAGCCAG	36
8	OPC-09	CTCACCGTCC	36
9	OPD-01	ACCGCGAAGG	36
10	OPD-02	GGACCCAACC	36
11	OPD-03	GTCGCCGTCA	36
12	OPD-04	TGAGCGGACA	36
13	OPD-05	TTGGCACGCG	36
14	OPD-06	GTGTGCCCCA	36
15	OPD-07	AGCGCCATTG	36
16	OPD- 09	CTCTGGAGAC	36
17	OPD- 11	AGCGCCATTG	36
18	OPD- 17	TTTCCCACGG	36
19	OPD- 20	ACCCGGTCAC	36
20	VR062	**F** CGAAGACGAAATCTGAAGACAA	59
		R TTACTTCTCCCAGCACTCCAAT	
21	VR0155	**F** AAGATCACACACAACCAACCC	59
		R AATTAGTTCCACAGGCCAGATT	
22	VR0222	**F** TCTCTTCTCTCTTCTCTCTTCTTCTC	59
		R TTCTGTCTGAGGCTATGTTGGT	
23	VR0223	**F** GCGTGATCGAGGCAGACTAT	59
		R GTGGGTAGCTCGGTAATAGCAC	
24	VR0304	**F** GAAGCGAAGAAGCCATAGAAAA	59
		R CCTCACACACAACACAACAGAA	
25	Phi057	**F** CTCATCAGTGCCGTCGTCAT	64
		R CAGTCGCAAGAAACCGTTGCC	
26	Phi112	**F** TGCCCTGCAGGTTCACATTGAGT	64
		R AGGAGTACGCTTGGATGCTCTTC	
27	Umc1066	**F** ATGGAGCACGTCATCTCAATGG	64
		R AGCAGCAGCAACGTCTATGACACT	
28	FER	**F** TCGCAAAGTTGCCAGTCAGT	59
		R TAGAAGGAAGGAGGGCATG	
29	URP- 2	**F** GTGTGCGATCAGTTGCTGGG	65
		R CCCAGCAACTGATCGCACAC	
30	ISSR842	GAGAGAGAGAGAGAGACG	45
31	ISSR856	ACACACACACACACACTA	45
32	ISSR836	AGAGAGAGAGAGAGAGCA	45
33	ISSR857	ACACACACACACACACCG	55

Fig. 1. Dendrogram 15 mungbean genotypes based on UPGMA method using RAPD, SSR and ISSR primers

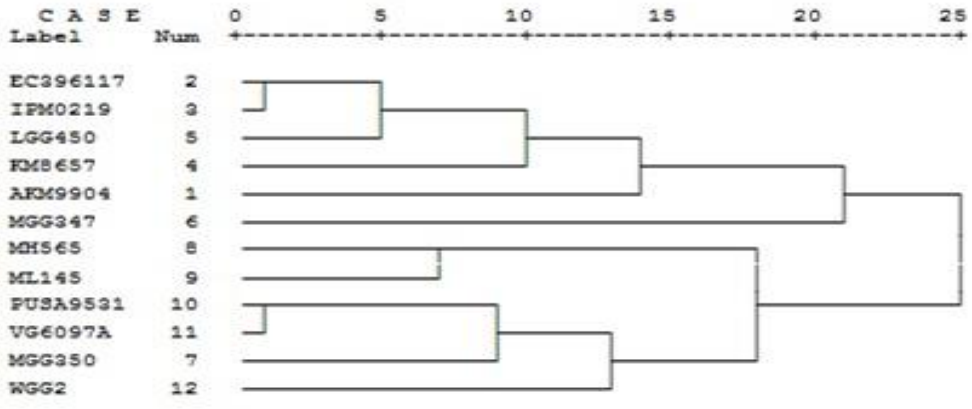

Table 2 Characteristics of amplification products obtained from eighteen primers

S.No.	Primer	Total No. of bands	Total No. of polymorphic bands	% of polymorphism
1	OPA 1	6	4	66
2	OPB 1	4	2	50
3	OPB 6	15	12	80
4	OPB 12	17	14	82
5	OPD 2	10	6	60
6	OPD 3	8	5	62
7	OPD 6	9	9	100
8	OPD 7	10	8	80
9	OPD 11	7	6	85
10	OPD 20	8	4	50
11	VR067	2	1	50
12	VR0155	4	4	100
13	VR0222	9	7	77
14	VR0223	8	5	62
15	VR0304	7	4	57
16	ISSR842	13	9	69
17	ISSR856	15	11	73
18	ISSR857	7	6	85

Table. 3 Clustering of genotypes using dendrogram

S. No.	Clusters	No. of genotypes	Genotype names
1	Cluster I	4	EC-396117, IPM-02-19, LGG 450 and KM-8-657
2	Cluster II	1	AKM 9904
3	Cluster III	1	MGG 347
4	Cluster IV	2	MH-565 and ML-145
5	Cluster V	4	PUSA 9531, VG-6197A, MGG 350 and WGG 2

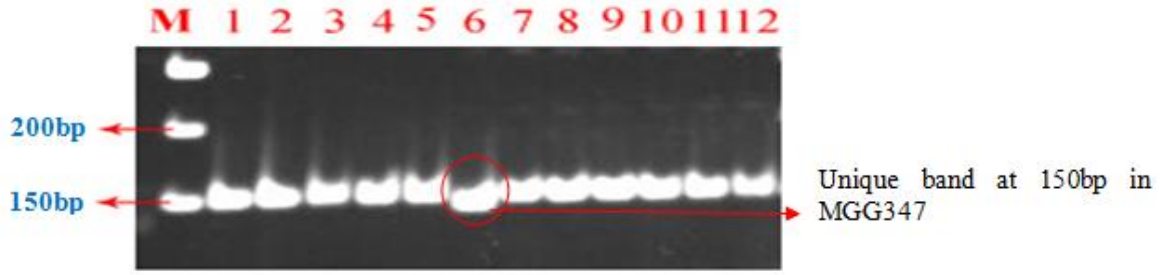

Fig 2. SSR gel profile of twelve mungbean genotypes using VR0155 primer

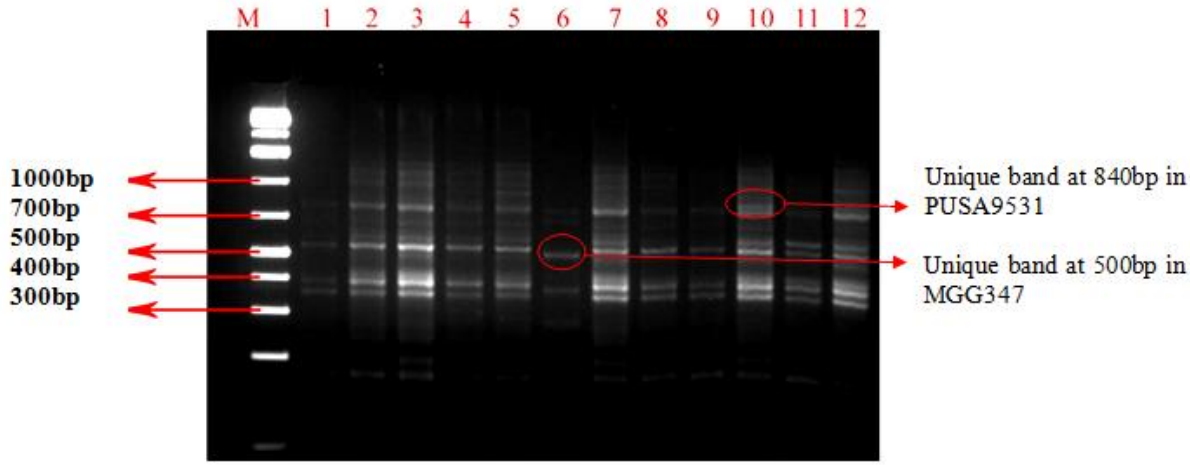

Fig 3. ISSR gel profile of twelve mungbean genotypes using ISSR842 primer

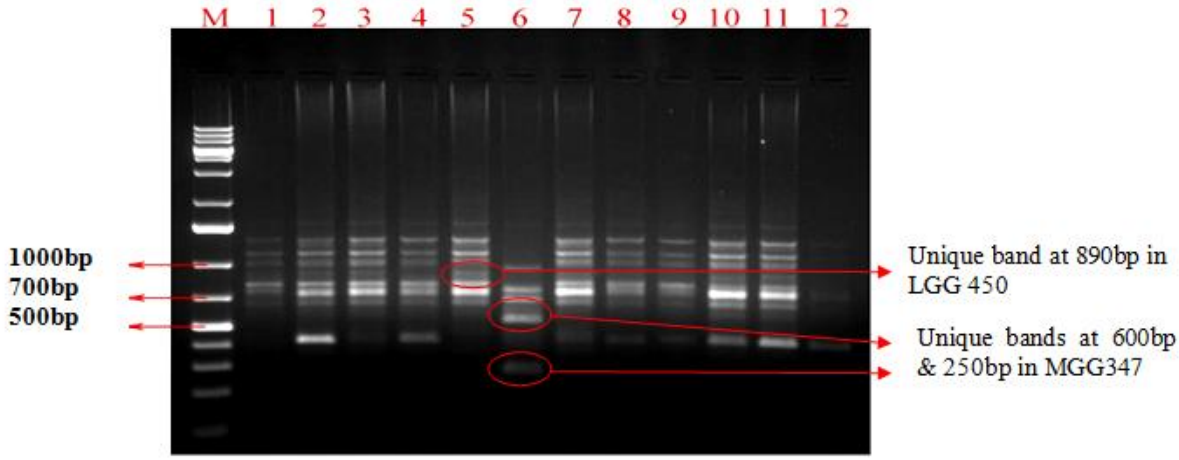

Fig 4. RAPD gel profile of twelve mungbean genotypes using OPB12 primer

1. AKM 9904	**2. EC-396117**	**3. IPM-02-19**	**4. KM-8-657**
5. LGG 450	**6. MGG 347**	**7. MGG 350**	**8. MH 565**
9. ML-145	**10. PUSA 9531**	**11. VG-6197A**	**12. WGG 2**