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Studies on for genetic polymorphism of 16 Odisha landraces of *Vigna mungo* (L.) Hepper for YMV resistance as revealed by RAPD marker

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ABSTRACT

Genetic variability of 16 blackgram (*Vigna mungo*) genotypes of the family Leguminaceae, one of the important pulses of Odisha, was was studied through RAPD markers. DNA marker analysis revealed an average variation of 42.61% polymorphism in banding patterns of the total score of 176 RAPD amplicons. Cluster analysis of RAPD markers showed that all the genotypes of blackgram studied from Odisha belongs to two distinct clusters i.e. cluster I and II. All the landraces showed narrow genetic diversity except cluster-II (BG-3 of Mahimunda and BG-4 of Berhampur). Among the 10 genotypes of cluster I, BG9, BG10 and BG11 showed close affinity collected from Panthnagar having highest divergence in BG9 which could be used as a potential source of genetically diverse germplasm of blackgram for improved crop breeding for developing YMV resistance.

1. Introduction

Vigna mungo commonly known as black gram of the family Leguminaceae is a bean grown in the Indian subcontinent. Blackgram is very nutritious and play an important role in human diet. However, crop loss in this crop is high due to Mungbean yellow mosaic virus (MYMV) caused by geminivirus transmitted through whitefly (*Bemicia tabaci*). The blackgram, mungbean and soybean are most seriously affected leguminous crops as reported by Verma and Singh (1986). MYMV disease symptoms shows irregular patches of yellow green spots on older leaves and young leaves of susceptible infected plants shows complete yellowish leaves.

Blackgram commonly known as Urd bean in India is a self pollinating diploid (2n=2x=22) annual crop having a genome size of approx. 574 Mbp. Improvement of blackgram against MYMV through breeding is quite difficult as MYMV is transmitted through white fly without s uniform procedure. Mostly, resistance is controlled by recessive genes. The limited success has made with development of disease free and high yielding varieties through conventional breeding. The cytogenetic and genetic mechanisms controlling the organization and evolution of genomes of *Vigna* species are not clearly understood. Hence, selection of land races and local genotypes using molecular markers linked to resistant genes should be an alternative and effective approach to overcome the inaccuracy in only field evaluation of morphological traits (Tanksley *et al*., 1989). Studies on disease resistant gene have indicated a high level of polymorphism and presence of SSRs at certain loci (Yu *et al*., 1996). SSR are highly polymorphic which are abundant as well as dispersed throughout the genome. Present study deals with SSR marker analysis in 15 land races of *Vigna mungo* collected from different localities of Odisha to estimate an preliminary study for YMV resistance genotypes if any suitable for future use in breeding purpose and MYMV marker development.

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2. Materials and Methods

2.1 Plant materials

Sixteen landraces of blackgram including local and established varieties were collected from different agro-ecological parts of Odisha (Table 1). Information regarding the varieties was collected using rural appraisal. The landraces were maintained in green house and experimental nursery of Department of Botany, Utkal University using standard nursery practices.

2.2. DNA isolation, purification and quantification

Nuclear DNA was isolated from young and juvenile leaves (2 g) of one month old seedlings using CTAB method (Saghai-Maroof *et al.*, 1984). The leaf collected from each landraces was ground to a fine powder form using liquid nitrogen with the help of motor and pestle and suspended in six volume of CTAB extraction buffer [2% CTAB,100mM Tris HCl (pH 8.0), 20 mM EDTA, 1.5M NaCl and 2% â-mercaptoethanol (v/v)]. The suspension was incubated in water bath at 60°C for 1-1.5h. An equal volume of chloroform:isomayl alcohol (24:1) was added to the suspension bringing down to room temperature and gently emulsify for 15 min and finally centrifused at 12000 rpm for 20 min at 4°C. The aqueous phase was transferred into new sterile centrifuge tube and DNA was precipitated with two third of its volume of chilled isopropanol. DNA was spooled or the pellet was transferred to 70% ethanol and washed and dried in room temperature. Dried DNA was dissolved in $T_{10}E_1$ (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and treated with RNase A (Sigma 10 µm ml^{-1}) at 37°C for 1h. The DNA was purified by phenol: chloroform (1:1) extraction and precipitated in ethanol (2.5 volumes) in the presence of 0.3 M sodium acetate (pH 5.2). The DNA was spooled out, washed in 70 % ethanol, air-dried, dissolved in $T_{10}E_1$ buffer and its concentration was determined and diluted to 25 ng μl^{-1} using $T_{10}E_1$ buffer for use as a template for RAPD analysis with different primers (Tables 2).

2.3 PCR analysis and RAPD profile

RAPD profiles were generated by using primers (Table 2) in Polymerase Chain Reaction (PCR) following the standard protocol of Williams *et al.* (1990). A total 20 RAPD primers were used to generate amplified bands in PCR. Each amplification reaction mixture contained 25 ng of template DNA, 200 µM of each dNTPs, 25 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 10 × Taq buffer (50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl2, pH 9.0) in a final reaction volume of 25 µl. The reaction was carried out in a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer, USA). The first cycle consisted of denaturation (94°C) for 5 min, primer

Table 1

List of blackgram landraces collected from different districts of Odisha for genetic analysis.

Sl No.	Accession No.	Local Name	Place	District	Latitude/Longitude
1.	BG1	Bhawanipatna local	Bhawanipatna	Kalahandi	19.91°N 83.12°E
2.	BG2	Badamba local	Badamba	Cuttack	20.31°N 85.47°E
3.	BG3	Mahimunda local	Mahimunda	Balangir	20.72°N 83.48°E
4.	BG4	B-3-8-8 (Prasad)	OUAT, Berhampur	Berhampur	19.32°N 84.78°E
5.	BG5	Ujjala	OUAT, Berhampur	Berhampur	19.32°N 84.78°E
6.	BG6	Nayagarh local	Nayagarh	Nayagarh	20.12°N 85.10°E
7.	BG7	Berhampur local	Berhampur	Berhampur	19.32°N 84.78°E
8.	BG8	Kothagarh local	Kothagarh	Kandhamal	20.47°N 84.23°E
9.	BG9	PU-31	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
10.	BG10	PU-40	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
11.	BG11	PU-19	Pantnagar	Udham Singh Nagar	28.98°N 79.40°E
12.	BG12	Badachana local	Badachana	Jajpur	20.85°N 86.33°E
13.	BG13	Ranipeta local	Ranipeta	Gajapati	18.88°N 84.20°E
14.	BG14	Keonjhar Pejua	Keonjhar	Keonjhar	21.63°N 85.60°E
15.	BG15	Similiguda local	Similiguda	Koraput	18.85°N 82.73°E
16.	BG16	Soroda local	Soroda	Mayurbhanj	21.93°N 86.73°E

Table 2
RAPD profile and DNA polymorphism of 16 blackgram landraces.

Primer name	Sequence (5'- 3')	No. of bands amplified	No. of polymorphic bands	Polymorphic %	Size range of amplified bands
OPA08	GTCACGTAGG	12	5	41.66	200-800
OPA11	CAATCGCCGT	10	6	60.00	300-1200
OPA14	TCTGTGCTGG	8	5	62.50	200-1100
OPD-08	GTGTCCCCCA	7	4	57.14	300-800
OPD-12	CACCGTATCC	10	4	40.00	200-900
OPE03	CCAGATGCAC	6	2	33.33	200–1000
OPF08	GGGATATCGG	7	2	28.57	200–1200
OPF10	GGAAGCTTGG	4	2	50.00	200–900
OPF12	ACGGTACCAG	12	5	41.66	300–1300
OPF13	GGCTGCAGAA	10	3	30.00	250–1100
OPF16	GGAGTACTGG	5	2	40.00	300–800
OPW02	ACCCCGCCAA	11	3	27.27	150–1300
OPW03	GTCCGGAGTG	10	4	40.00	200–1350
OPW05	GGCGGATAAG	9	5	55.55	200–1100
OPW06	AGGCCCGATG	5	2	40.00	250–900
OPW08	GACTGCCTCT	11	4	36.36	250–1050
OPW11	CTGATGCGTG	10	4	57.14	300–900
OPN-04	GACCGACCCA	14	6	42.85	200-800
OPN-11	TCGCCGCAAA	8	5	62.50	100-1200
OPN-15	CAGCGACTGT	7	2	28.57	400-1300
		176	75	42.61	

annealing (42°C) for 1 min and DNA polymerization (72°C) for 2 min. In the next 40 cycles the period of denaturation was maintained at 92 °C for 1 min while the primer annealing and DNA polymerization was same as in the first cycle. The last cycle consisted of only primer extension (72°C) for 8 min. The amplified products were stored at 4°C and separated by electrophoresis on 1.5 % agarose gel in 1×TAE buffer for 2 h at 60 V. Gene Ruler 100 bp DNA ladder plus (MBI Fermantas, Lithuania) was used as the size standard to determine the size of the polymorphic fragments. DNA fragments were visualized by staining the gel with ethidium bromide and images were documented using Gel Doc G700 (BioRad, USA). Only those amplification products that appeared consistently in three replications were scored for further analysis.

2.4 Data analysis

The visualization of presence or absence of the bands was taken into consideration in RAPD analysis, but the differences in their intensity were ignored. Amplified bands of RAPD primers were scored as present (1) or absent (0) in each landraces for each set of primers and only clear and reproducible bands were used in this study. A binary matrix was obtained from the RAPD profile using NTSYS-pc programme (Rohlf, 2008). Binary matrix was transformed into a similarity matrix using Jaccard's coefficient. From this matrix a phylogenetic dendrogram was obtained by cluster analysis following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Sneath and Sokal, 1973).

2.5 Principle component analysis (PCA)

The Jaccard's similarity matrix was subjected to principle component analysis. This coordination method makes use of multidimensional solution of the observed relationship. PCA resolves complex relationships into a function of fewer and simpler factor. Principal components were derived for each landraces using eigen vectors and

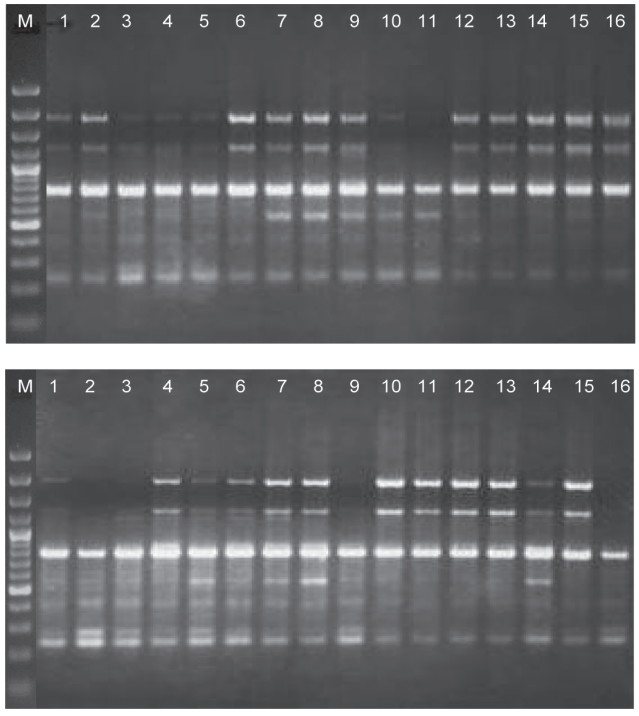

Fig. 1. RAPD profile of 16 land races of blackgram amplified with OPA-08 primer.

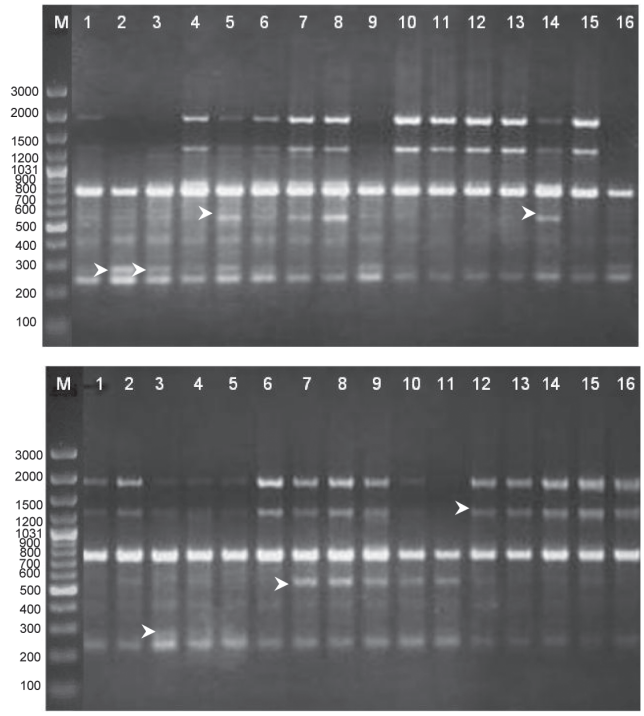

Fig. 2. RAPD profile of 16 land races of blackgram amplified with OPN-04 primer.

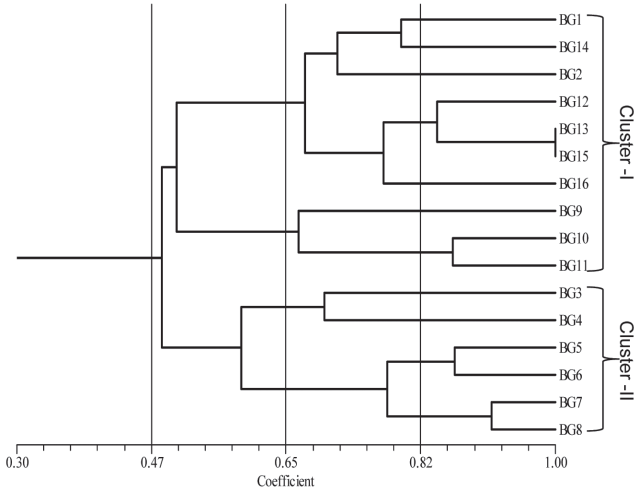

Fig. 3. Dendrogram of 16 land race of blackgram constructed using UPGMA based on Jacard's similarity coefficients for the RAPD data set.

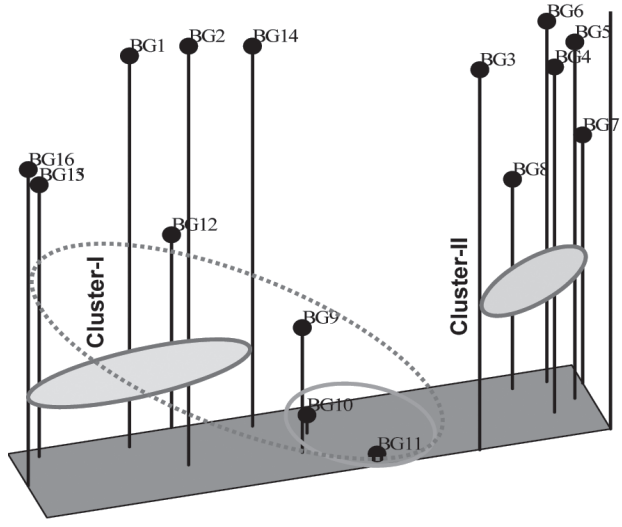

Fig. 4. Three-dimensional plot of principal component analysis of 16 genotypes of blackgram with different genotypes showing genetic diversity.

eigen values extracted from a correlation matrix among the markers that was obtained from a standardized data matrix. All the above analyses were carried out using NTSYS-pc (Version 2.02e, Applied Biostatistics) program. Bootstrap analysis (1000 iterations) of the binary data was performed using the WINBOOT programme (Yap and Nilsson, 1996) to determine the confidence limits of the UPGMA based dendrograms and boot-strap of 50 % majority rule consensus tree was constructed. Average discrimination coefficients (D) of each RAPD primers were estimated for all the 48 landraces with band differences ranging from one to five using the Power Marker (Liu and Muse, 2005) software tool. In this, data matrix was derived from the distance or similarities between the operational taxonomic units.

3. Results and discussion

Genetic variability was found to be associated with morphological traits. Morphological markers are offen

influenced by environmental factors. So, RAPD markers have been used in many instances to assess the genetic variability in genetic resource utilization (Panigrahi *et al.,* 2015; Shelke and Das, 2015; Sahu *et al.*, 2016). Total 176 RAPD amplicons were generated by 20 primers, of which 75 (42.61%) were polymorphic (Tables 2, Figs. 1 and 2). Each primer gave 4 to 14 amplified bands by OPF-10 and OPN-04 primers respectively having a size range from 100 bp to 2000 bp (Table 2). Genetic similarity on the basis of RAPD analysis for 16 varieties of blackgram ranged from 2% 65%. The results indicate that most divergent group were BG-5 to BG-8 from the rest of the varieties. The unique markers obtained from different varieties could be used to develop variety specific SCAR markers for identification of genotypes. In this study numbers of unique bands were discovered by RAPD marker (Figs. 1 and 2) which could be cloned and utilized for the preparation of variety specific marker (SCAR) and physical localization on chromosomes through FISH. Present study of RAPD marker throws light on phylogeny and classification of local landraces of blackgram germplasms for further study, breeding practices and management of germplasms. $RAPD_{800}$ was the common monomorphic band in OPA8 and OPN4 primer as well as some other primers. Some of the polymorphic bands like $RAPD_{600}$ in BG5, BG6, BG7, BG8 and BG-14 of 'Ujala', 'Nayagarh local', 'Berhampur local', 'Kothagarh local' and 'Keonjhar Pejua' are special marker bands amplified in OPA8 primer. OPN14 similarly produced some marker band like $RAPD_{2000}$ and $RAPD_{1400}$ in some of the land races (Fig. 4, arrow heads). $RAPD_{600}$ produced by BG7, BG8, BG9, BG10, BG11 in OPN4 was also found to be landrace specific. A unique band of $RAPD_{250}$ in 'Mahimunda local' of Balangir district was landrace specific in OPN4 primer. Prasanthi *et al.* (2011) reported RAPD marker based SCAR marker development in blackgram for large-scale application in marker-assisted breeding for YMV resistance. This involves the characterization of the linked maker and the design of locus-specific primers. The conversion of a linked marker to SCAR has been reported in common bean (Melotto *et al.,* 1996), rice (Naqvi and Chattoo, 1996), blackgram (Prasanthi *et al.,* 2011) and tomato (Zhag and Stommet, 2001). Gupta *et al.* (2013) also used F_2 population to tag and map the MYMIV resistance gene using SSR markers in blackgram.

The results presented here showed that 16 land races are considerably different genetically in cluster distribution that depends mainly on the genomic constituents of the genotypes. The close similarities of BG13 (Ranipeta local) and BG15 (Simliguda local) might be of common origin as these two land races are found in nearby districts. Single largest Cluster-I was produced with 10 land races (Fig. 3) out of which BG13 and BG15 (Gajapati and Similiguda) were having very high genetic similarity. Cluster-II formed with rest 6 land races out of which BG3 and BG4 showed more genetic distance from the other 4 land races (Fig. 3). PCA analysis showed that in the Cluster-IBG9, BG10 and Bg11 of Panthnagar are of same genetic background with a high genetic variability (Fig. 4). As such Cluster-I showed high genetic variability than Cluster-II. Saraswathi *et al.* (2011) and Shelke and Das (2015) reported also intra-group diversity among banana using IRAP and RAPD marker systems. The amplified products could be tested at different chromosomal sites among divergent species to check the common ancestor (Cuadrado, 2002; Achrem, 2006). This RAPD polymorphism could be due to deletion and/or amplification from the pre-existing sequences during varietal evolution. Very similar conclusions can be drawn from SSR-based studies (Shang, 2006) and ISSR-based studies (Ren, 2011). A recombinant inbred line (RIL) mapping population (F8) was generated by crossing *Vigna mungo* (cv. TU 94-2) with *Vigna mungo* var. *silvestris* and screened for mungbean yellow mosaic virus (MYMV) resistance. The inter simple sequence repeat (ISSR) marker technique was employed to identify markers linked to the MYMV resistance gene and one ISSR marker was identified as tightly linked to the MYMV resistant gene at 6.8 cM by Anjum *et al*. (2010). The ISSR technique has been used in tagging disease resistant genes in a number of crops (Reddy, *et al.,* 2002). ISSRs have better capacity to reveal polymorphism and offer great potential to determine intra- and inter-genomic diversity when compared with other arbitrary primers like RAPDs (Zietkiewicz, *et al.,* 1994; Souframanien and Gopalakrishna, 2006). A major obstacle is the lack of high resolution genetic markers for blackgram for development of YMV markers. The linkage mapping in black gram can be made possible using genetic markers developed from other related legumes. BAC library for *Vigna* species, except only one on mungbean (Miyagi *et al*., 2004), is one of the constrains in developing markers for disease resistance. Efforts are needed to develop expressed sequence tag (EST) libraries which offer important information for species that have not been sequenced and are a central source of gene based markers and single nucleotide polymorphism (SNP) or indel polymorphisms (Galeano *et al*., 2009). EST–based markers are valuable because they represent sequences that are transcribed and therefore, can potentially be associated with phenotypic differences. Generation of expressed sequence tag (EST) databases for the development of genic microsatellite markers might overcome the paucity of the polymorphic markers in black gram for developing MYMIV resistant genes. A single dominant gene controls the MYMIV resistance in blackgram genotype DPU 88-31 was reported for MYMIV resistance

gene using SSR markers (Gupta *et al.,* 2013). Morphologically distantly related and DNA based resistant varieties obtained from the cluster analysis could be used for plant breeding in black gram to develop high yielding disease resistant varieties.

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