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# "Investgation of Molecular Variability on MYMV Resistant and Susceptible Blackgram Specie Using SSR Marker"

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## Abstract:

Blackgram (*Vigna mungo* (L.) Hepper) is one of the major pulse crops of the tropics and sub tropics. It is the third major pulse crop cultivated in the Indian subcontinent. Pulses and grain legumes are major sources of dietary protein. Besides various biotic and abiotic constraints, viral diseases mostly yellow mosaic disease is the prime threat for extensive economic losses in areas of production. The advancements in the field of biotechnology and molecular biology such as marker assisted selection and genetic transformation can be utilized in developing MYMV resistance uradbeans. Various molecular markers have been used for the molecular analysis of grain legumes. Among different DNA markers, microsatellites (or) Simple Sequence Repeats (SSRs) have occupied a vital place due to their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage. Molecular markers and genetic linkage maps are essential for molecular breeding in any crop.

Our investigation was made to analyze the genetic diversity of 18 genotypes based on morphop-hysiological attributes. However, molecular markers are reported to be more advantageous over morphological traits in analyzing genetic diversity. Similarity coefficients for the 18 blackgram accessions based on 8 SSR markers. Pair-wise association among blackgram accessions, showed the highest similarity value **0.667** and the lowest Distance value **0.167**. The Cophenetic Correlation among 18 blackgram verities is 0.72731. The highest polymorphism was observed in primer VR135.

**Keywords:** Blackgram, MYMV, SSR, Polymorphism, Cophenetic Correlation.

## Introduction:

Blackgram (*Vigna mungo* (L.) Hepper) (2n=22) is one of the most highly valuable pulse crop, cultivated in almost all parts of India. It is a good source of easily digestible proteins, carbohydrates and other nutritional factors. Besides various biotic and abiotic constraints, viral diseases mostly yellow mosaic disease is the prime threat for extensive economic losses in areas of production. The advancements in the field of biotechnology and molecular biology such as marker assisted selection and genetic transformation can be utilized in developing MYMV resistance uradbeans. Blackgram is an important legume crop of the family leguminaceae and it is grown mainly in Indian subcontinent. On comparison with other pulse crop, blackgram is highly priced. It is considered as rich source of protein, potassium, iron, calcium, thymine (B1), niacin (B3), riboflavin (B2) and it is nutritious for human diet**.** It also helps in fixing atmospheric nitrogen to the soil **[1,4].**

In India, blackgram cultivation is followed not only in kharif season, it can also be grown in *Rabi* and summer seasons. Among various diseases infecting and reducing yield of black gram, yellow mosaic disease caused by mung bean yellow mosaic virus is the crucial one. MYMV belonging to Gemini viridae family and begomovirus group can affect crop yield upto 100 percent under higher incidence **[1,3].** MYMV in India cannot be transmitted by mechanical means and it can easily be transmitted by whitefly *Bemisia tabaci*. MYMV is highly infectious to legume crops such as black gram, mung bean, pigeon pea, French bean, soya bean causing symptoms like yellow flecks on leaves alternating with green patches. Upon severe infection, leaves become completely yellow and produce lesser flowers and pods. Due to non-availability of resistant varieties, cultivation of black gram crop land is diverted to other cereal crops cultivation and for MYMV management in urdbean production, breeding with the resistant cultivars is effective which ecofriendly is also. It is essential to find more number of resistant varieties which performs well at all growing seasons and hence to identify MYMV resistant urdbean cultivars several attempts have been made by researchers. In view of the above facts, the present study was targeted to screen the MYMV resistant blackgram varieties under natural condition **[2-4].**

Blackgram (*Vigna mungo* (L.) Hepper) also known as Uradbean, is one of the important pulse crops of India. India is the largest producer and also consumer of blackgram. It has surely marked itself as the most popular pulse crop and can be most consequently referred to as the “king of the pulses” due to its delicious taste and numerous other nutritional qualities. Blackgram is superb combination of all nutrients, which contains proteins (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins (Karamany 2006). Being a good leguminous crop, it is itself a mini-fertilizer depository, as it has special characteristics of maintaining and restoring soil fertility through fixing atmospheric nitrogen in symbiotic association with *Rhizobium* bacteria, present in the root nodules. It is short duration pulse crop usually flowering within 30-50 days of sowing and maturing within 60-90 days **[5,2].** Among various biotic and abiotic yield limiting factors, mungbean yellow mosaic disease (MYMD) caused by mungbean yellow

mosaic virus (MYMV) is the most destructive limiting factor in blackgram. Infection of MYMV may cause up to 85–100% yield loss in uradbean. The virus is transmitted by white flies (*Bemisia tabaci*). Various molecular markers have been used for the molecular analysis of grain legumes. Among different DNA markers, microsatellites (or) Simple Sequence Repeats (SSRs) have occupied a vital place due to their reproducibility, multi-allelic nature, co-dominant inheritance, relative abundance and good genome coverage. Molecular markers and genetic linkage maps are essential for molecular breeding in any crop. Such tools would speed up the process of introgression of useful traits into preferred varieties. Keeping in view of the above statements, the present study was undertaken to identify molecular markers linked to yellow mosaic virus resistance through bulk segregant analysis (BSA) in blackgram **[6-10].**

## Materials & Methods:

### Plant material

The identified resistant and susceptible parents for yellow mosaic virus i.e., T-9 and LBG-759 respectively were procured from Agriculture College Rewa (M.P.). A cross was made betweenT9 and LBG 759, F2 mapping population was developed from this cross was used for screening against YMV disease incidence. A total of 18 diverse black gram genotypes differing for their response to YMV disease were used in the study. The details of the genotypes used in the study are given in Table No. 1. The material was screened for the YMV reaction in the field under natural conditions over a period of two years. Under field conditions, resistant plants did not show any yellowing of leaves or pods during the growth period, while susceptible plants showed yellowing on the leaves and pods. Normal cultural practices were followed, except that there was no insecticide spraying. LBG17, a highly susceptible cultivar to YMV, was used as an insect refuge after every five rows. Genomic DNA was extracted from seedlings using DNeasy Plant Mini kit (Qiagen, USA) using the manufacturer's protocol and DNA concentration was determined on Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA). The DNA samples were diluted to 10 ng µl-1 for PCR amplification. PCR amplification: A total of 10 molecular markers reported to be linked to YMV resistance in black gram and mungbean were used for the validation study. The PCR amplification were performed in a 25 µl reaction volume containing 10 mM Tris - HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 0.01% gelatin, 0.2 mM each of dNTP, 20 pmoles of RAPD primer, 50 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase (Fermentas Life Sciences). Amplifications were performed in an Eppendorf Master Cycler (Eppendorf, Hamburg, Germany). Amplification conditions were an initial denaturation at 94oC for 4 min and 40 cycles at 94oC for 1 min, 37-60oC (depending on marker type) for 1min, 72oC for 1 min followed by 7 min at 72 oC. The annealing temperatures of different markers used in the study are given in Table 2. Amplified products were separated on 2% agarose gel in 1X TBE (Tris-Borate-EDTA) buffer. SSR products were resolved on 4% Metaphor agarose gel. The gels were stained with 0.5 µg/ml ethidium bromide solution and were photographed on a gel image analysis system (Syngene, U.K.).

**Table No. 1**

**Different genotype of Blackgram**

| S.No. | Genotype |
|---|---|
| BG1. | TU94-2 |
| BG 2. | Pant-U19 |
| BG 3. | KU 96-3 |
| BG 4. | Pusa-3 |
| BG 5. | TU-40 |
| BG 6. | Local Variety-1 |
| BG 7. | PU-31 |
| BG 8. | DPU88-31 |
| BG 9. | PU02-43 |
| BG 10. | IPU94-1 |
| BG 11. | PU07-3 |
| BG 12. | EC-168200 |
| BG 13. | TAU-1 |
| BG 14. | T-9 |
| BG 15. | LBG-17 |
| BG 16. | TPU-4 |
| BG 17. | EC-168058 |
| BG 18. | Local Variety-2 |

**Analysis of genetic diversity based on molecular markers (SSR):**

Genetic diversity studies reveal the genetic distance amongst the germplasm which is very much helpful in study of evolutionary relationship and formulating strategies for utilization of germplasm for future crop improvement programme. Uradbean is a crop of global importance including the country of India. Although, Uradbean is largely an introduced crop in India, a large number of Uradbean germplasm are now available in India. The crop offers great commercial significance in central India too. Although, the crop is not grown in commercial scale in this region, a lot of native genotypes are grown under wild and cultivated form in the region. As a part of the present study, an investigation was made to analyze the genetic diversity of 18 genotypes based on morphophysiological attributes. However, molecular markers are reported to be more advantageous over morphological traits in analyzing genetic diversity. Effects of the environment influencing the diversity study can be minimized by the use of molecular markers. Also, molecular markers offer more accurate diversity analysis being independent of developmental stage of crop under study. With the application of more reliable DNA markers e.g. Simple Sequence Repeats, the diversity study has become more precise. In order to supplement the information generated from the morphological data, the second objective was formulated to study the genetic diversity of 44 Uradbean genotypes based on SSR markers.

## Isolation of genomic DNA:

### Material

1. Leaf Sample: Leaf samples for extraction of DNA was collected from the same forty four Uradbean genotype set used for the first objective under present investigation. Genomic DNA was extracted from healthy leaves of 15-20 days old Uradbean seedling under study.

**2.** Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction Buffer (100 ml)

**Table No. 2**

**Composition**

| Components | Concentration | Volume | $P^H$ |
|---|---|---|---|
| Tris Chloride | 1M | 20 | 7.0 |
| EDTA | 0.5M | 4 | 8.0 |
| NaCl | 5M | 28 | |
| Beta- Marcaptoethenol | 4% | 2 | |
| CTAB | 10% | 20 | |
| Sterile Water | | 26 | |

3. Tris EDTA (TE) Buffer (PH 8.0)

TRISHC1 10 mM

EDTA 1 mM

4. 1N HC1

5. 1NNaOH

6. Chloroform: Isoamyl mixture (24:1)

7. RNase A (lmg/ml)

8. Ethanol [100% concentrated (ice cold) and -70% (normal)

9. Simple Sequence Repeat markers: A set of 30 SSR markers were used for genetic diversity analysis. The markers were selected covering the whole genome of Uradbean. The sequences of the markers were downloaded from the ‘soybase’ (http://www.soybase.org) and synthesized and procured through vendors (Sigmaaldrich. com). The list of marker with base sequences and linkage group is given in table no 3.3.

**Table No. 3: Selected Primer sequence and annealing temperature**

| S.No. | Marker | Anealing Temperature | Primer Sequence |
|---|---|---|---|
| **1.** | **VR073** | **59** | F: GGTAGTTCATTTCGGCCACTT<br>R: GGTAGTTCATTTCGGCCACTT |
| **2.** | **VR102** | **58** | F: CATGTGAGCTACCCTTTCAACA<br>R: CAAGGACTGCTATATCCAAGGC |
| **3.** | **VR216** | **58** | F: TTCCCTGTGTCCTTATATGTCC<br>R- GAGGATAGTGAATTTTGAAGGC |
| **4.** | **MB 13** | **57** | F – GCAGCAACAACAGCAACA<br>R- GCAGGTTTTGTGGCTCAG |
| **5.** | **MB 77** | **57** | F – GGAGAGGAAGGAACAGGG<br>R- GGCAGAGCATAAACATGGC |
| **6.** | **MB 87** | **57** | F – TCCCTTGTGGGAGATCCT<br>R- CTTTGCCACACTCCTTGC |
| **7.** | **VR 4** | **58** | F – TGGTTGGTTGGTTCACAAGA<br>R- CACGGGTTCTGTCTCCAATA |
| **8.** | **VR169** | **58** | F - GGAAGATAGCGGAGATGAAGAG<br>R- CACCATACACCATAACATTCCTG |
| | | | |

**Methods:**

Leaf samples were collected from 15-20 days Uradbean seedling in healthy trate. Leaves were washed properly first with tap water followed by rinsing with distilled water to remove the dirt and insects and dried using tissue paper. Genomic DNA was extracted from washed young leaves by following CTAB extraction protocol (Saghai - Maroof *et al.*, 1984). Prior to the extraction, the pestle and mortar, spatula, scissors and all glassware to be used were autoclaved. About 1.0-1.5 g of young leaf from each sample were weighed, cut into small pieces and then were transferred to mortar. Using CTAB extraction buffer (400 **µl** +400 **µl** +400 **µl**), the leaf samples were grinded finely. The grinded mass was transferred to a 2.0 mL micro centrifug tube and kept in one hour incubation in water bath at 65° C. After the incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly for forty five minutes by gentle inversion. The contents were centrifuged at 12000 revolutions per minute (rpm) for 20 minutes. The supernatant (top aqueous layer) was carefully transferred to new micro centrifuge tube. 5 µl of RNAse was added to each tube and kept for incubation at 37° C for half an hour. After incubation, the contents were centrifuged at 10000 rpm for 10 minutes. The supernatant was transferred to new micro-centrifoge tube while discarding the pellet. Approximately 400 **µl** volume of ice cold Ethanol (100 *%)* was added to it and mixed well and, kept in -20° C for overnight. The precipitated DNA was centrifuged at 10000 rpm for 10 minutes to form DNA pellet which was washed with Ethanol (70 %). Supernatant was decanted and the DNA pellet was air dried completely at room temperature. Depending upon the size of the pellet, DNA was dissolved in 7-200 **µl** of TE (PH 8.0) and stored at 4° C.

**Quality checking of DNA:**

DNA was checked for its purity and intactness by agarose gel electrophoresis. A part of DNA sample was run on 0.8 % agarose stained with Ethidium Bromide following standard protocol (Sambrook *et ah,* 1989) to estimate its quality. DNA bands were visualized in a gel documentation system (US Major Science).

**Quantification of DNA:**

Purity and quantification of the leaf-extracted DNAs was done using spectrophotometer (Systronics, Double beam spectrophotometer 2203). 10 **µl** of stock DNA was diluted with 1990 **µl** of sterile double distilled water and O.D. was taken at wavelength 260 nm and 280 nm. The ratio OD 260 / OD 280 was determined to assess the purity of the sample. The concentration of the template DNA in the sample was calculated using the following formula: DNA Concentration (pg/pl) =OD 260 x (Dilution factor) x 50 x 10"3

**Dilution of DNA:**

Based on the quantification data, part of each DNA sample was diluted with sterilized distilled water to yield a working concentration of 10 ng/**µl.** The diluted samples were stored at 4° C for immediate use, while the original undiluted DNA samples were kept for long term storage at -20° C.

**Polymerase chain reaction (PCR) analysis using SSR:**

A set of 10 SSR primers was selected covering the whole genome of Uradbean. The genomic DNA isolated from forty four Uradbean genotypes were amplified by using these 30 SSR markers. PCR amplifications of the DNA were done in a reaction volume of 20 **µl**. The reaction volume contained all the necessary components in right concentrations.

**Polymerase Chain Reaction mixture:**

| **Reagents** | **Manufacturer** | **Concentration** | **Quantity (µl)** |
|---|---|---|---|
| ***Taq* buffer A (with $MgCl_2$)** | **Biolit, SRL** | **10X** | **2.0** |
| **dNTFs** | **Do** | **10 mM** | **2.0** |
| **SSR primer (Forward)** | **Do** | **20 ng / µl** | **2.0** |
| **SSR primer (Reverse)** | **Do** | **20 ng/ µl** | **2.0** |
| ***Taq* DNA Polymerase(3U/ µl)** | **Do** | **3 U / µl** | **0.3** |
| **Double distilled H2O** | **Do** | | **6.7** |
| **Template DNA** | **Do** | **20 ng/ µl** | **5.0** |
| **Total** | | | **20.0** |

The amplification of DNA sample was carried out in Thermo cycler (Sure cycler 8800, Agilient Technology) programmed in initial denaturation at 94° C for two min followed by 39 cycles consisting of denaturation, primer annealing and extension at 94° C, 45-55° C and 72° C, respectively for one minute each. The final extension step was performed at 72° C for seven minutes.

### 3.2.6. Agarose gel electrophoresis:

**Material:**

A. High resolution metaphore agarose

B. Tris Acetate EDTA (TAE) buffer

C. Ethidium Bromide (10 mg /ml)

D. 6X DNA loading dye

**Method:**

Metaphor agarose electrophoresis was used to study the polymorphism showed by SSR marker. Metaphor agarose had been used by a number of workers to study genetic diversity in an easier and economical way (Asif *et al.,* 2008, Legesse *et ah,* **2007,** Ghosh *et al.,* 2014). A 3 % metaphor gel was prepared by melting 15 g of agarose in 500 mL of IX TAE buffer in a microwave for approximately 2 minutes. Prepared gel was allowed to cool for a couple of minutes and then 20 pi of EtBr (10 mg/ml) was added. Gel was casted using a supplied tray and comb and the gel was allowed to set for a minimum of 20 minutes at room temperature on a flat surface. 3 pi of 6X loading dye was added to each amplified PCR product and was loaded in prepared gel with a reference 100 bp ladder. The electrophoresis was carried out at 80 V for 1.5 hours. The assembly was switched off once the dye reaches the 2/3rd the length of the gel. The gel was exposed to UV transilluminator (US Major Science) for visualizing the bands and photographs were taken by using Gel Documentation System (US Major Science), and subsequently used for scoring leading to polymorphic study.

**Analysis of Polymorphism:**

A manual scoring system was used to score the SSR alleles. Clearly resolved, unambiguous polymorphic bands were scored visually for their presence or absence. The scores were obtained in the form of a matrix with one (1) and (0), indicating the presence and absence of bands in each genotype, respectively. The genotypes showing two allelic bands with equal intensity were considered as heterozygous for that locus. Polymorphism information content (PIC) for each SSR marker was calculated based on the formula Hj = 1 Epi, where pi is the allele frequency for the i-th allele (Senior and Heun, 1993). Principle Component Analysis (PCA) was carried out to support the pattern of genetic diversity providing a graphical representation of diversity among the genotypes in the form of 2-D image. Jaccard's similarity coefficient (J) was used to calculate the genetic similarities among pair wise comparison of genotypes based on SSR following the formula **J=Nn / (Nn + Nio+Noi)** (Jaccard, 1908) where, Nn is the number of bands present in both genotypes; Niois the number of bands present in one genotype and Noi is the number of bands present in the other genotype. Similarity matrix was generated using the software NTSYS 2.0. Cluster analysis was done using similarity coefficient and dendrogram was constructed following UPGMA approach.

## Results:

We studied physiological and genetic variability in Blackgram and resistance ability to MYMV (Mungbean yellow mosaic virus) in vindhyan region of Madhya Pradesh. We identified resistance gene to MYMV (Mungbean yellow mosaic virus) in Blackgram. In present study attempts were made to develop a population from the cross between LBG-759 (MYMV susceptible parent) and T9 (MYMV resistant parent). MYMV resistant and susceptible parents were selected and used for identifying molecular markers linked to MYMV resistance.

### Identification of Different Blackgram Specie:

First we identified YMV susceptible and YMV Resistance plant of blackgram on the basis of physical appearance of leaves. A susceptible plant showing yellow spot in leaves whereas no any spot seen in resistant plant **(see figure no. 1).**

**A. YMV susceptible**

**B. YMV Resistance**

Figure No. 1: A. YMV susceptible Blackgram plant, YMV Resistance Blackgram plant.

**Table No. 4**
**Screening of Different Blackgram Specie**

| S.No. | Genotype | Reaction to YMV |
|---|---|---|
| **BG1.** | **TU94-2** | Resistant |
| **BG 2.** | **Pant-U19** | Resistant |
| **BG 3.** | **KU 96-3** | Resistant |
| **BG 4.** | **Pusa-3** | Resistant |
| **BG 5.** | **TU-40** | Resistant |
| **BG 6.** | **Local Variety-1** | Resistant |
| **BG 7.** | **PU-31** | Resistant |
| **BG 8.** | **DPU88-31** | Resistant |
| **BG 9.** | **PU02-43** | Resistant |
| **BG 10.** | **IPU94-1** | Resistant |
| **BG 11.** | **PU07-3** | Resistant |
| **BG 12.** | **EC-168200** | Resistant |
| **BG 13.** | **TAU-1** | Susceptible |
| **BG 14.** | **T-9** | Susceptible |
| **BG 15.** | **LBG-17** | Susceptible |
| **BG 16.** | **TPU-4** | Susceptible |
| **BG 17.** | **EC-168058** | Susceptible |
| **BG 18.** | **Local Variety-2** | Susceptible |
| | | |

**Molecular Characterization of Different Black gram Specie:**

Our result from SSR molecular analysis characterize YMV Resistant and susceptible blackgram plant. Many commercial black gram varieties are susceptible to YMV and there is a need to identify tightly linked molecular markers that could facilitate the transfer of the resistant genes in to popular cultivars using marker assisted breeding. In this study, ISSR molecular markers which identified as linked to YMV resistance in blackgram. SSR marker SSR8111357 reported to be linked to YMV resistance (6.8 cM) in black gram (Souframanien and Gopalakrishna, 2006) amplified the 1357 bp marker fragment in 12 of the 18 black gram genotypes and marker fragment was absent in all six YMV susceptible black gram genotypes.

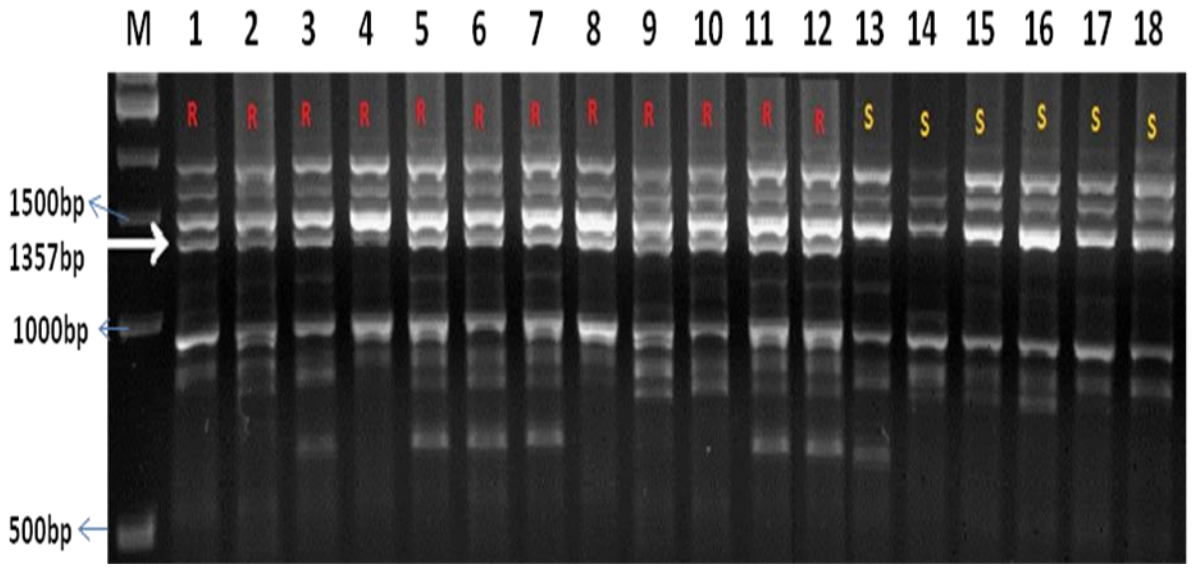



Figure No. 2: Amplification profile of SSR marker SSR8111357 on 18 blackgram genotypes. Lane M: 1Kb DNA MW marker; Lane 1: TU94-2 ; 2: Pant-U19 ; 3: KU 96-3 ; 4: Pusa-3 ; 5: TU-40 ; 6: Local Variety-1; 7: PU-31 ; 8: DPU88-31 ; 9: PU02-43 ; 10: IPU94-1 ; 11: PU07-3 ; 12: EC-168200 ; 13: TAU-1 ; 14: T-9; 15: LBG-17 ; 16: TPU-4 ;17:EC-168058 ; 18: Local Variety-2. R: YMV resistant and S: YMV susceptible. *Arrow* indicates the 1357 bp fragment linked to MYMV resistance.

**Analysis of genetic diversity based on molecular markers (SSR):**

**Analysis of polymorphism**

Our result from polymorphism analysis revealed genetical variability. Presence of DNA in the sample was tested through electrophoresis in 0.8 % Agarose gel. Clear and thick bands in the gel indicated presence of ample DNA in isolated samples. The quantity of DNA was determined by taking Optical density of samples at 260 nm in spectrophotometer. The quantity of DNA in all the 18 samples varied from 700-1400 ng /pi which were more than enough for amplification through PCR. Polymorphism information content (PIC) was calculated to determine the level of polymorphism among Simple Sequence Repeat (SSR) primers. In this study, all the 8 SSR markers got amplified and produced clear and scorable bands. Out the 8 SSR markers, two markers VR135 (PIC=0.8141) and VR323 (PIC=0.6571) were found to be maximum PIC value. Polymorphism information content (PIC) is a measure of degree of informativeness of molecular markers. The number of alleles with respect to each SSR marker alongwith the PIC value is presented in Table no. 4.2. Number of alleles ranged from two to seven with an average of 3.5 allele per locus. Highest allelic variability was observed in VR135 with 7 allele and VR323 with 4 allele. MB14, MB87, VR9, VR163, VR155 and VR216 was the least variable locus each with three alleles whereas VR216 with 2 allele. The SSR marker profiles of 18 Blackgram genotypes generated by the primer VR323, MB87, VR135, and VR216 are presented in Figure No. 4.3,4.4,4.5 and 4.6 respectively. The higher the PIC value, the more informative is the SSR marker.PIC values of the markers ranged from 0.3743 to 0.8141 with an average of 0.5583.

**Table No. 5:**

**Number of allele and PIC of 10 polymorphic SSR markers**

| S.No. | Marker | No. Of Allele | Heterozygosity | PIC |
|---|---|---|---|---|
| **1.** | **VR323** | 4 | 0.7099 | 0.6571 |
| **2.** | **MB14** | 3 | 0.6349 | 0.5629 |
| **3.** | **MB87** | 3 | 0.5034 | 0.4160 |
| **4.** | **VR9** | 3 | 0.6427 | 0.5668 |
| **5.** | **VR135** | 7 | 0.8352 | 0.8141 |
| **6.** | **VR163** | 3 | 0.5864 | 0.5009 |
| **7.** | **VR155** | 3 | 0.6484 | 0.5749 |
| **8.** | **VR216** | 2 | 0.4986 | 0.3743 |
| Average of Allele per locus = 3.5, average PIC= 0.5583 | | | | |

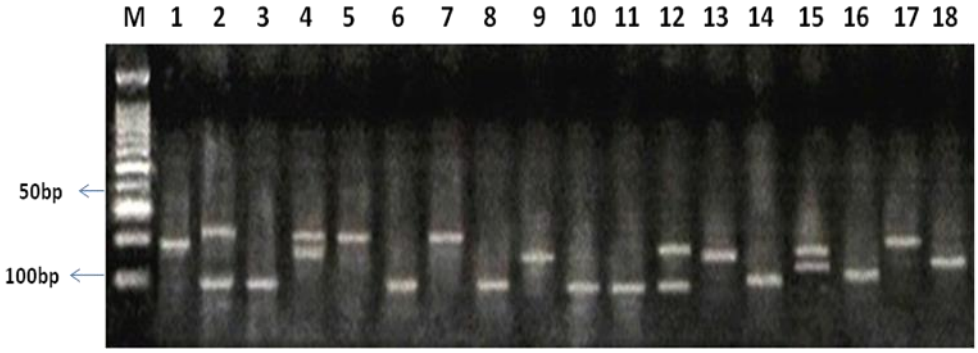



Figure No. 3: PCR amplification with VR323 primer.

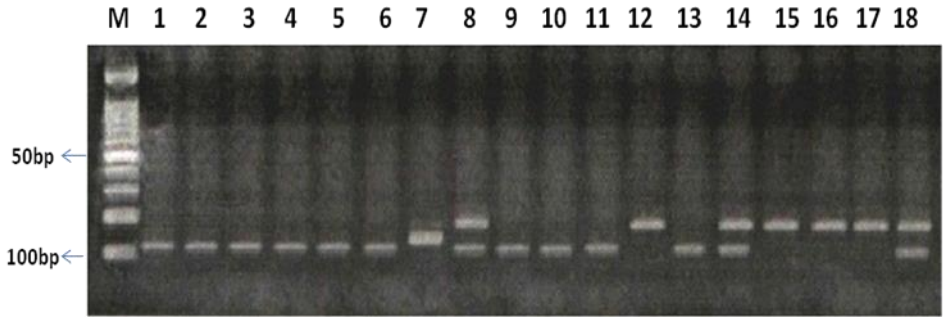



Figure No. 4: PCR amplification with MB87 primer.

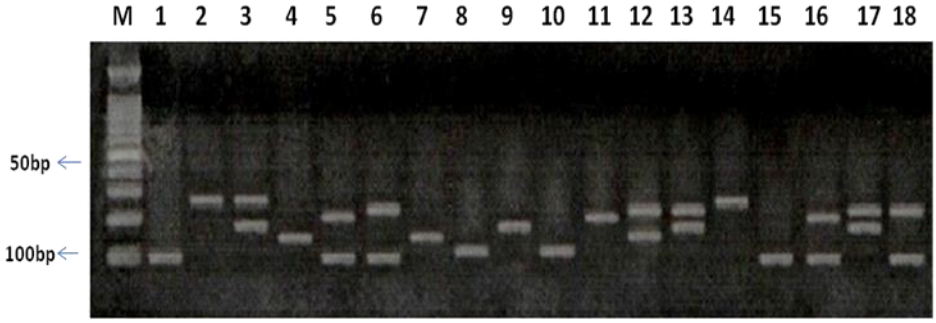



Figure No.5: PCR amplification with VR135primer.

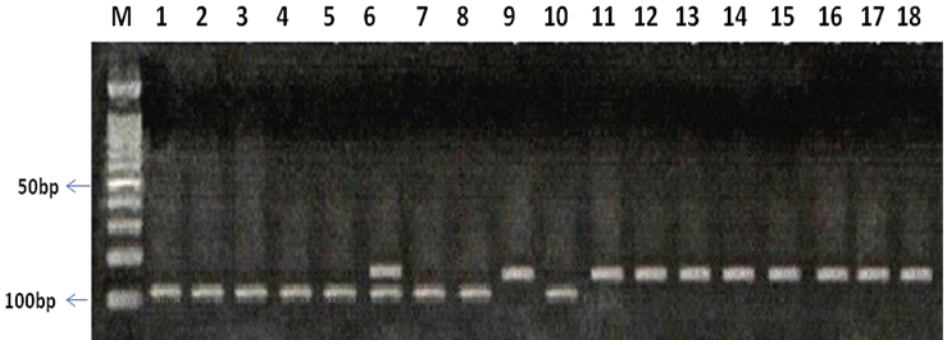



Figure No 6: PCR amplification with VR216 primer.

**Molecular Variability Analysis:**

The UPGMA is the simplest method of tree construction. It was originally developed for constructing taxonomic phenograms, i.e. trees that reflect the genotypic similarities between OTUs, but it can also be used to construct phylogenetic trees if the rates of evolution are approximately constant among the different lineages. For this purpose the number of observed nucleotide or amino-acid substitutions can be used. UPGMA employs a sequential clustering algorithm, in which local topological relationships are identifeid in order of similarity, and the phylogenetic tree is build in a stepwise manner. We first identify from among all the OTUs the two OTUs that are most similar to each other and then treat these as a new single OTU. Such a OTU is referred to as a composite OTU. Subsequently from among the new group of OTUs we identify the pair with the highest similarity, and so on, until we are left with only two UTUs.

Similarity coefficients for the 18 blackgram accessions based on 8 SSR markers. Pair-wise association among lackgram accessions, showed the highest similarity value **0.667** and the lowest Distance value **0.167**. The Cophenetic Correlation among 18 blackgram verities are 0.72731. The highest polymorphism was observed in primer VR135. The dendrogram showing the genetic relationships among 18 blackgram accessions based on SSR markers is presented in NCSS dendrogram report. The dendrogram showed three major clusters. Cluster A (primary cluster) consisting Pusa-3, Pant-U19, LBG-17 and EC 168200. Cluster B consisted of 14 verities of blackgram that are polyembryonic in SSR marker. Cluster B further grouped in to two clusters C (PU31, TAU-1, Local Varity-2 and TU-40)

and D. Cluster D again grouped into cluster E (TPU-4, PU02-43, KU96-3, EC168058 and Local Varity-1) and F (IPU94-1, PU07-3, TU94-2, T-9 and DPU88-31).

**Table No. 4.3**
**Polymorphic locus in Blackgram varieties:**

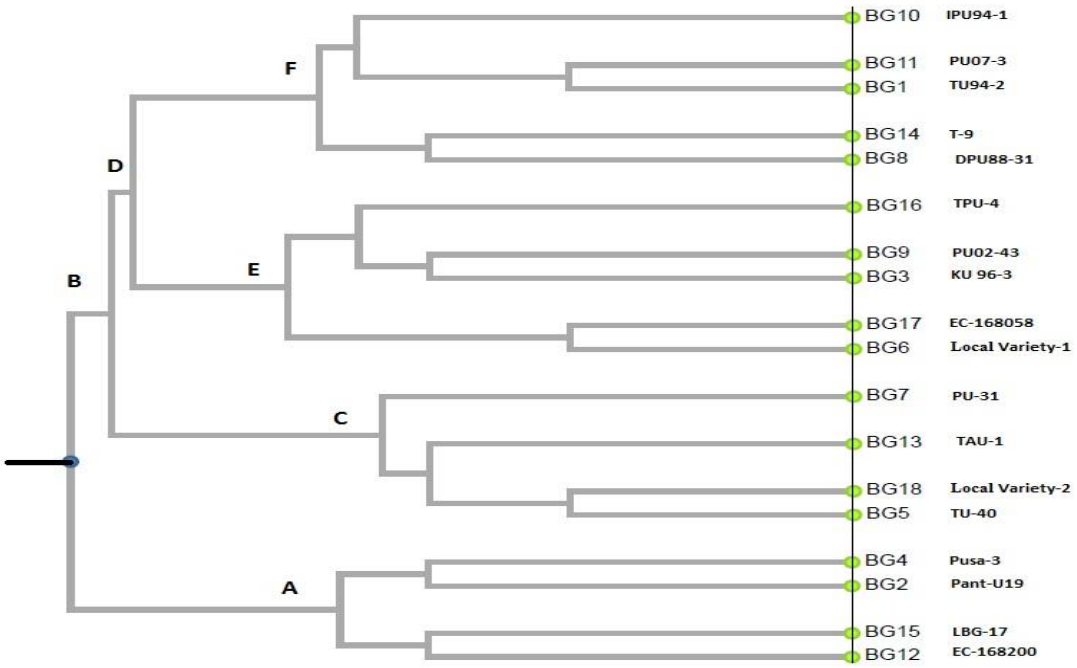
| S.No. | Variety | VR323 | MB14 | MB87 | VR9 | VR135 | VR163 | VR155 | VR216 |
|---|---|---|---|---|---|---|---|---|---|
| BG1. | TU94-2 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| BG 2. | Pant-U19 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BG 3. | KU 96-3 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 |
| BG 4. | Pusa-3 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| BG 5. | TU-40 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 |
| BG 6. | Local Variety-1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 |
| BG 7. | PU-31 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| BG 8. | DPU88-31 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| BG 9. | PU02-43 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| BG 10. | IPU94-1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| BG 11. | PU07-3 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 0 |
| BG 12. | EC-168200 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| BG 13. | TAU-1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| BG 14. | T-9 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| BG 15. | LBG-17 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| BG 16. | TPU-4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| BG 17. | EC-168058 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| BG 18. | Local Variety-2 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |

**1= polymorphic, 0= single allele**

**Table No. 4.4 Similarity index among all blackgram variety**

| | BG 1 | BG 2 | BG 3 | BG 4 | BG 5 | BG 6 | BG 7 | BG8 | BG 9 | BG 10 | BG1 1 | BG1 2 | BG1 3 | BG1 4 | BG1 5 | BG1 6 | BG1 7 | BG1 8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BG1** | 1 | 0.000 | 0.333 | 0.000 | 0.000 | 0.250 | 0.000 | 0.500 | 0.000 | 0.500 | 0.667 | 0.200 | 0.000 | 0.333 | 0.333 | 0.000 | 0.333 | 0.000 |
| **BG2** | | 1 | 0.000 | 0.500 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.250 | 0.000 | 0.000 | 0.500 | 0.000 | 0.000 | 0.000 |
| **BG3** | | | 1 | 0.000 | 0.333 | 0.250 | 0.000 | 0.200 | 0.500 | 0.000 | 0.250 | 0.500 | 0.000 | 0.000 | 0.333 | 0.333 | 0.333 | 0.250 |
| **BG4** | | | | 1 | 0.000 | 0.000 | 0.000 | 0.200 | 0.000 | 0.000 | 0.000 | 0.500 | 0.000 | 0.000 | 0.333 | 0.333 | 0.000 | 0.000 |
| **BG5** | | | | | 1 | 0.250 | 0.500 | 0.000 | 0.500 | 0.000 | 0.250 | 0.200 | 0.333 | 0.000 | 0.000 | 0.333 | 0.333 | 0.667 |
| **BG6** | | | | | | 1 | 0.000 | 0.167 | 0.333 | 0.333 | 0.200 | 0.167 | 0.000 | 0.250 | 0.000 | 0.250 | 0.667 | 0.200 |
| **BG7** | | | | | | | 1 | 0.000 | 0.000 | 0.000 | 0.333 | 0.000 | 0.500 | 0.000 | 0.000 | 0.000 | 0.000 | 0.333 |
| **BG8** | | | | | | | | 1 | 0.000 | 0.250 | 0.400 | 0.333 | 0.200 | 0.500 | 0.200 | 0.200 | 0.200 | 0.167 |
| **BG9** | | | | | | | | | 1 | 0.000 | 0.000 | 0.250 | 0.000 | 0.000 | 0.000 | 0.500 | 0.500 | 0.333 |
| **BG10** | | | | | | | | | | 1 | 0.333 | 0.000 | 0.000 | 0.500 | 0.000 | 0.000 | 0.500 | 0.000 |
| **BG11** | | | | | | | | | | | 1 | 0.167 | 0.250 | 0.250 | 0.250 | 0.000 | 0.250 | 0.200 |
| **BG12** | | | | | | | | | | | | 1 | 0.000 | 0.000 | 0.500 | 0.500 | 0.200 | 0.167 |
| **BG13** | | | | | | | | | | | | | 1 | 0.333 | 0.000 | 0.000 | 0.000 | 0.667 |
| **BG14** | | | | | | | | | | | | | | 1 | 0.000 | 0.000 | 0.333 | 0.250 |
| **BG15** | | | | | | | | | | | | | | | 1 | 0.000 | 0.000 | 0.000 |
| **BG16** | | | | | | | | | | | | | | | | 1 | 0.333 | 0.250 |
| **BG17** | | | | | | | | | | | | | | | | | 1 | 0.250 |
| **BG18** | | | | | | | | | | | | | | | | | | 1 |

**Cophenetic Correlation Coefficient (CP) = 0.72731**




Graph No. 4.1: UPGMA dendrogram of Blackgram germplasm based on SSR marker.

**Discussion:**

**B**lackgram or Urdbean (Vigna mungo (L.) Hepper) is a highly self-pollinated crop with cliestogamy up to 42%. Urdbean is grown all over the South East Asia. In India it is mostly grown as a kharif crop. In the past, remarkable progress has been made towards the development of high yielding, stress resistant and input responsive varieties by utilizing the available germplasm. The impact of these varieties has been well realized in their crop productivity. The present review highlights the past, present and future importance of improving high yield in black gram through different breeding strategies viz., selection, hybridization, mutation and other molecular breeding approaches. But alien gene transfer for several traits such as photo and thermo sensitive, erect plant types stand still. Heterotic studies in blackgram, the important genetic mechanism operates to bring superiority in F1 hybrid than their parents. Heterosis has been of immense economic value in agriculture and has important implications regarding the fitness and fecundity of individuals in natural populations **[11,9].**Considering blackgram (Vigna mungo L.), a pulse crop which is self-pollinated, little work has been done on heterosis. This genetic tool is the basic mechanism in developing blackgram cultivars with high yielding potentials. The increase in blackgram production volume comes mainly from the increase in blackgram cultivated area. A possible breakthrough for this production limitation is to exploit hybrid vigor of the F1 for possible production of hybrid varieties. The magnitude of hybrid vigor is normally presented in terms of heterosis (superiority of the F1 hybrid over its parental mean) and heterobeltiosis (superiority of the F1 hybrid over its better parent. The results on heterosis so far in blackgram were encouraging and still there a scope to utilize this genetic phenomenon to develop new cultivars superior than existing. Hence this review illustrates the facts and proof for different research on black gram and the future prospects **[10-15].**

Black gram (Vignamungo L. Hepper) is a pulse crop and used as a protein rich food in human diet. India is the largest producer of black gram. A primary gene centre of black gram is found in India, thus many landraces of black gram is available in this region, which can be used as parental lines for improvement of black gram against YMV infection. In the present study 32 black gram genotypes was screened to identify the YMV infection resistant and susceptible genotypes through field experiment. The four genotypes of black gram i.e, UPU 8335, IPU 99-25, PGRU-95004 and SPS-43 were identified as highly YMV resistant, whereas the four genotypes i.e. IPU 2K-99-226, IPU 99-3, IPU 99-235 and NDU 94-10 were identified as highly susceptible genotypes. The present study will be helpful to suggest the YMV resistant genotypes for farmers in north India anduseful to select the parental lines for development of YMV resistant black gram varieties through crop improvement program **[16-21].**

We studied physiological and genetic variability in Blackgram and resistance ability to MYMV (Mungbean yellow mosaic virus) in vindhyan region of Madhya Pradesh. We identified resistance gene to MYMV (Mungbean yellow mosaic virus) in Blackgram. In present study attempts were made to develop a population from the cross between LBG-759 (MYMV susceptible parent) and T9 (MYMV resistant parent). MYMV resistant and susceptible parents were selected and used for identifying molecular markers linked to MYMV resistance. Urdbean Leaf Crinkle Virus (ULCV) is an important

viral disease which is most serious in case of susceptible genotypes and favorable environmental conditions.

An efficient procedure for sprouted seed method of agroinoculation in black gram crop with infectious dimer agro clones of mungbean yellow mosaic virus (MYMV) DNA-A and DNA-B has been developed using black gram susceptible genotype PBG-1. Different parameters like Agrobacterium cell density, concentration of acetosyringone, inoculation buffer, incubation time, incubation temperature and place of pinpricking were standardized for maximum infectivity of MYMV virus in agroinoculated plants **[22-24].** The Agrobacterium cell density at OD600 value 0.8 gave high percentage of virus infection followed by OD600 value 1 and 0.5. It was observed that high percentage of virus infection gave in 100 µm concentration of acetosyringone followed by 80 µm and 150 µm concentrations of acetosyringone. Agroinoculated blackgram seeds were incubated at three incubation times (1hour, 5hours and overnight) and 80-100% percentage of infection was observed at all three incubation times, similarly incubation temperatures (25oC, 28oC and 37oC were evaluated for maximum percentage of infection and 82% of infection was observed at incubation temperature 28oC followed by 25oC and37oC, even poor germination was noticed at incubation temperature 37oC. First time we evaluated the place of pinpricking on hypocotyl region for maximum virus infection and found that pinpricking around the hypocotyls gave high percentage of virus infection (79%) in agro-inoculated blackgram plants **[25-32].**

We identified YMV susceptible and YMV Resistance plant of blackgram on the basis of physical appearance of leaves. A susceptible plant showing yellow spot in leaves whereas no any spot seen in resistant plant. Our result from ISSR molecular analysis characterize YMV Resistant and susceptible blackgram plant. Many commercial black gram varieties are susceptible to YMV and there is a need to identify tightly linked molecular markers that could facilitate the transfer of the resistant genes in to popular cultivars using marker assisted breeding. In this study, ISSR molecular markers which identified as linked to YMV resistance in blackgram. ISSR marker ISSR8111357 reported to be linked to YMV resistance (6.8 cM) in black gram amplified the 1357 bp marker fragment in 12 of the 18 black gram genotypes and marker fragment was absent in all six YMV susceptible black gram genotypes **[33,12].**

YMV transmitted by whitefly is the major disease in pulses that causes the severe yield loss in India as well as in other blackgram growing countries in Asia. Molecular markers linked with YMV can improve the process of identification of resistant genotypes. In the present investigation Simple Sequence Repeats (SSR) and Bulk segregant analysis (BSA) techniques were used to analyse the F2 individuals of T9 (resistant) × LBG-759 (susceptible) to screen and identify the yellow mosaic virus (YMV) resistant gene in urdbean. Two DNA bulks, namely resistant and susceptible bulks were setup by pooling equal amount of DNA from ten extreme phenotypes, resistance and susceptible plants. Parental survey study was carried out by using 59 SSR primers. This study revealed that 12 SSR markers showed polymorphism between the parents. These polymorphic markers were utilized for bulk segregant analysis (BSA). Among the polymorphic SSR markers, one primer viz., VR9 were able to distinguish the resistant and susceptible bulks and individuals indicating that this marker is tightly linked to yellow mosaic virus

resistance gene and report of YMV-resistance linked marker in blackgram. This marker VR9 could be utilized in the marker assisted breeding programme **[34,22].**

Mungbean [Vigna radiata (L.) Wilczek] is an important legume which can be grown in varying environmental conditions, during all three crop seasons viz., kharif, rabi and spring/summer in India, as sole or inter crop for grain and green manure. It is an excellent source of easily digestible proteins with low flatulence, which complements the staple rice diet in Asia. Mungbean Yellow Mosaic Disease (MYMD) caused by whitefly (Bemisia tabaci) transmitted mungbean Yellow Mosaic Virus (MYMV) is an important constraint of mungbean. To fulfill future demands, there is a need to use molecular marker technology and other biotechnological interventions. Hence, Bulked Segregant Analysis (BSA) with a Random Amplified Polymorphic DNA (RAPD) marker technique was used to analyze the F2 **[35-40].**

Our result from polymorphism analysis revealed genetical variability. Polymorphism information content (PIC) was calculated to determine the level of polymorphism among Simple Sequence Repeat (SSR) primers. In this study, all the 8 SSR markers got amplified and produced clear and scorable bands. Out the 8 SSR markers, two markers VR135 (PIC=0.8141) and VR323 (PIC=0.6571) were found to be maximum PIC value. Polymorphism information content (PIC) is a measure of degree of informativeness of molecular markers. The number of alleles with respect to each SSR marker alongwith the PIC value is presented in Table no. 4.2. Number of alleles ranged from two to seven with an average of 3.5 allele per locus. Highest allelic variability was observed in VR135 with 7 allele and VR323 with 4 allele. MB14, MB87, VR9, VR163, VR155 and VR216 was the least variable locus each with three alleles whereas VR216 with 2 allele. The SSR marker profiles of 18 Blackgram genotypes generated by the primer VR323, MB87, VR135, and VR216 are presented in Figure No. 4.3,4.4,4.5 and 4.6 respectively. The higher the PIC value, the more informative is the SSR marker.PIC values of the markers ranged from 0.3743 to 0.8141 with an average of 0.5583.

Yellow mosaic disease (YMD) is caused by Mungbean yellow mosaic virus (MYMV) major disease of mungbean in India as well as in other mungbean growing countries in Asia. In the present investigation Simple Sequence Repeats (SSR) and Bulk segregant analysis (BSA) techniques were used to analyse the F2 individuals of susceptible VBN (Gg) 2 × resistant KMG 189 to screen and identify the Mungbean yellow mosaic virus (MYMV) resistant gene in mungbean. These SSR primers can produce polymorphism between parents and are not able to distinguish the bulks. This attributes the fact that these SSR primers are heterologous probes developed in azuki bean which can produce polymorphism between parents and are not able to distinguish the bulks **[41-47].** Black gram (Vigna mungo L. Hepper), is an extensively studied food crop which is affected by many abiotic and biotic factors, especially diseases. The yield potential of Black gram is shallow due to lack of genetic variability and biotic stress susceptibility. The highest similarity coefficient was observed between IC-145202 and IC- 164118 (0.921), while lowest similarity was between PU- 31 and IC-145202 (0.572). The genetic diversity obtained in this study along with disease analysis suggests PU31as a useful variety for the development

of markers linked to MYMV, UCLV, wilt and powdery mildew resistance by marker-assisted back cross breeding and facilitates the production of crosses with multiple disease resistance **[48-53].**

Our statistical study of UPGMA reveals similarity among all genotype. The UPGMA is the simplest method of tree construction. It was originally developed for constructing taxonomic phenograms, i.e. trees that reflect the genotypic similarities between OTUs, but it can also be used to construct phylogenetic trees if the rates of evolution are approximately constant among the different lineages. For this purpose the number of observed nucleotide or amino-acid substitutions can be used. UPGMA employs a sequential clustering algorithm, in which local topological relationships are identifeid in order of similarity, and the phylogenetic tree is build in a stepwise manner. Similarity coefficients for the 18 blackgram accessions based on 8 SSR markers. Pair-wise association among lackgram accessions, showed the highest similarity value **0.667** and the lowest Distance value **0.167**. The Cophenetic Correlation among 18 blackgram verities are 0.72731. The highest polymorphism was observed in primer VR135. The dendrogram showing the genetic relationships among 18 blackgram accessions based on SSR markers is presented in NCSS dendrogram report. The dendrogram showed three major clusters. Cluster A (primary cluster) consisting Pusa-3, Pant-U19, LBG-17 and EC 168200. Cluster B consisted of 14 verities of blackgram that are polyembryonic in SSR marker. Cluster B further grouped in to two clusters C (PU31, TAU-1, Local Varity-2 and TU-40) and D. Cluster D again grouped into cluster E (TPU-4, PU02-43, KU96-3, EC168058 and Local Varity-1) and F (IPU94-1, PU07-3, TU94-2, T-9 and DPU88-31).

Yellow mosaic virus (YMV) disease is a serious disease which affects the black gram productivity. In this study, 10 molecular markers reported to be linked to YMV resistance in black gram and mungbean were validated on 19 diverse black gram genotypes for their utility in marker assisted selection. Three molecular markers (ISSR8111357, YMV1-FR and CEDG180) differentiated the YMV resistant and susceptible black gram genotypes. Other seven molecular markers were either monomorphic or failed to amplify the marker fragment in black gram genotypes **[54-58].** A 136-bp allele of Simple Sequence Repeat (SSR) marker CEDG180 linked to YMV resistance was amplified in the aforementioned four genotypes in addition to PU 31. ISSR8111357 and CEDG180 are located on different linkage groups in the black gram genetic linkage map, suggesting that two independent resistance genes may be governing resistance to YMV in Indian black gram cultivars with marker ISSR8111357 linked to one resistance gene and marker CEDG180 linked to other resistance gene. Therefore, both markers can be used for marker assisted selection of YMV resistance. Among the resistant genotypes, PU 31 was the only genotype where both markers were amplified suggesting that PU 31 might be carrying both YMV resistance genes. Therefore, genotype PU 31 would be a valuable donor of YMV resistance and should be actively used in black gram breeding programs for incorporating YMV resistance **[59-61].**

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