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Screening of Cowpea (*Vigna unguiculata* (L). Walp) Genotypes for Resistance to Yellow Mosaic Disease Caused by CYMV and BCMV using Specific SSR Markers

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ABSTRACT

The research was conducted during August to November, 2020 at the Department of Plant Biotechnology and Dryland Farming Unit, University of Agricultural Sciences (UAS), Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru, Karnataka, India to validate the identified SSR markers associated with resistance to yellow mosaic diseases of cowpea using different genotypes. 120 germplasm lines cowpea were used for the study including two check varieties (IC-202781 and V-585). Disease scoring scale by Diwakar and Mali (1976) was used for the disease scoring. Among 120 genotypes, 52 genotypes with moderately resistance, 28 genotypes with no disease symptoms, 15 genotypes with resistance, 14 genotypes with moderately susceptible, 11 genotypes with susceptible and no highly susceptible genotypes were observed. These germplasms were screened using four simple sequences repeat markers (SSR) specific to Bean common mosaic virus and four SSR markers specific to cowpea yellow mosaic virus were selected from the existing databases. Out of the four SSR marker linked to BCMV tested for validation, only three markers (M15, M80, and Y 96) gave the desired amplification. Single marker analysis (SMA) revealed that out of four SSR markers, MA15 marker explains 3.96% of phenotypic variation, among 4 SSR markers linked to CYMV, only two markers (VM1 and AG1/AF48383) gave the desired amplification. SMA revealed that out of two SSR markers, AG1/AF48383 marker explains 3.80% of phenotypic variation. The resistant genotypes identified in this investigation can be used in future breeding programmes for introgression of disease resistant traits in important competitive varieties in the market.

KEYWORDS: Cowpea, yellow mosaic disease, CPMV, BCMV, SSR markers

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1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L). Walp.) belongs to family Fabaceae is a warm season short day crop that adapts to high temperature and drought conditions (Fantaye et al., 2017). In India, area and production under cowpea is 3.9 million hectares and 2.21 mt respectively with the productivity of 683 kg ha^{-1}. Karnataka is one of the leading cowpea growing states in India covering 12% of area and 10 of the production. However, productivity of cowpea in Karnataka is very low (423 kg ha^{-1}) because of biotic and abiotic stresses (Dinesh et al., 2016). Among the diseases, viral diseases which co-evolve with cowpea are significantly affecting the yield in Asia, Africa and Latin America. More than 20 viruses have negative effects on cowpea production worldwide (Mbeyagala et al., 2014). Among which, CPMV is devasting and may cause 80 to 100% yield reductions. However, BCMV belonging to potyvirus group is major virus transmitted by aphids and seeds (Manjunatha et al., 2017). These viruses majorly cause yellow mosaic disease leading to mosaic and yield reductions up to 95%.

The family *Geminiviridae* comprised of nine genera, and the viruses are attributed to respective genus depending on its host, vector and genome arrangements (Varsani et al., 2017). Geminiviruses are transmitted by various types of insect (whiteflies, leafhoppers, treehoppers and aphids). Geminiviruses are plant pathogens causing economically important diseases in most tropical and subtropical regions of the world (Zerbini et al., 2017). Reprogramming of cowpea cellular metabolism was reported through Physiological, biochemical, and proteomic analyses at 2 and 6 days' post salt stress (DPS). Proteomic analysis showed differential contents of 403 and 330 proteins at 2 DPS and 6 DPS, respectively, out of 733 differentially abundant proteins between the two plant groups (Varela et al., 2019).

There is need for developing different ways to manage YMD and development of mungbean yellow mosaic virus (MYMV) resistant genotypes. Advanced molecular and biochemical approaches can be used for characterization of YMD resistance during plant-virus interactions (Mishra et al., 2020). The local isolates of CABMV and CPSMV was inoculated to sixteen cowpea genotypes/cultivars and identified resistant genotypes/cultivars that can be used in breeding programs (Guillermo et al., 2023). Development and introduction of cultivars resistant to the diseases is an economical and eco-friendly option to the resource poor cowpea farmers (Haggag et al., 2015). Conventional breeding of cowpea for resistance to cowpea mosaic virus disease has met limited success owing to cumbersome procedure for identifying and selecting resistant genotypes in breeding programmes (Narayana and Angamuthu, 2021). PCR amplifications confirmed the presence of virus for the DNA extracted from the seeds (with yellow patches on the seed coat) from MYMIV infected mungbean plants but no amplification was found in the seedlings of PCR positive seeds showed YMD is not seed-borne (Naimuddin et al., 2016). The seed borne nature of YMD was reported by Kothandaraman et al. (2016).

An improved consensus genetic linkage map has been developed and used to identify QTLs of additional traits. Development of single nucleotide polymorphism (SNP) genotyping is being streamlined to establish an efficient workflow supported by genotyping support service (GSS)-client interactions. Several cowpea breeding programs have been exploiting these resources to implement molecular breeding, especially for MARS and MABC, to accelerate cowpea variety improvement (Boukar et al., 2016). The DNA markers can be used as surrogate to identify disease resistant genotypes (Dinesh et al., 2018). Among the available DNA markers, microsatellites or simple sequence repeats (SSRs), have gained considerable importance in plant genetics and breeding. (Kalia et al., 2011). Identification of SSR markers linked to genomic regions controlling target traits and their validation are the prerequisites for using them in plant breeding (Kumar, 2016).

Screening of 100 genotypes against Bean common mosaic virus (BCMV) using artificial inoculation was reported which showed different disease reaction. 25 randomly selected resistance and susceptible cowpea genotypes were screened using 10SSR markers out of which only 4 primers were found to be polymorphic and they were used for characterization and finding out genetic relationship of cowpea genotypes (Manjunatha et al., 2017). The present study aims at validation of identified SSR markers associated with resistance to yellow mosaic diseases of cowpea using different genotypes.

2. MATERIALS AND METHODS

The research work was carried out at the Department of Plant Biotechnology and Dryland Farming Unit in collaboration with AICRP on Arid legumes, ZARS, University of Agricultural Sciences (UAS), Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru during the kharif season (August–November) of 2020. The experimental material comprised of a total of one hundred and twenty genotypes along with two check varieties (IC-202781 and V-585) grown in augmented block design. Geographical location: Bengaluru, Karnataka, India. 3 HJG+RWG, GKVK Campus road, Vignana Kendra, Bengaluru, Karnataka 560065, India, Latitude 13.081989°, Long 77.577251°.

2.1. Assessment of disease severity of different cowpea genotypes

The disease severity on each genotype was assessed regularly at 15 days' interval on ten randomly selected plants as per cent disease incidence. The genotypes were grouped into different categories as resistant or susceptible using the disease scoring scale as suggested by Diwakar and Mali (1976) (Table 1). Per cent disease incidence was calculated by using the following formula.

Percent disease incidence (%)=No. of plants infected/ T otal no. of plants observed×100

Table 1: Disease scoring scale by Diwakar and Mali (1976)

Scale	Description	Category
0	No visible symptoms	Immune
1	Plants showing disease symptoms of 1–5%	Resistant
2	Plants showing disease symptoms of >5–15%	Moderately resistant
3	Plants showing disease symptoms of >15–25%	Moderately susceptible
4	Plants showing disease symptoms of>25–50%	Susceptible
5	Plants showing disease symptoms more than 50%	Highly susceptible

2.2. Molecular characterization of cowpea genotypes using SSR markers

2.2.1. Genomic DNA isolation from cowpea genotypes

Leaf samples of fifteen days old seedlings of one hundred and twenty cowpea accessions were collected from field-grown plants. The young and healthy leaves were crushed using liquid nitrogen and DNA was extracted by the Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). The quality and quantity of DNA is assessed by loading DNA samples into 0.8% (w/v) agarose gel prepared in 1X TAE buffer containing 0.5 µl 10 ml^{-1} ethidium bromide followed by visualization and documentation using Alpha digidoc 1000 gel documentation system (Alpha Innotech Corporation, USA). Further, the quality of DNA was assessed by checking shearing of DNA and contamination with RNA. Total DNA was quantified by using the multi-plate reader and the DNA stocks of the samples were diluted to the required 40–50 ng μl^{-1}.

2.2.2. Molecular characterization of cowpea genotypes using SSR markers

Eight SSR markers were used in the present study and annealing temperatures were reconfirmed by using the gradient polymerase chain reaction (PCR) technique containing genomic DNA (1 µl $reaction^{-1}$), takara mixture (a thermo stable DNA polymerase, dNTPs, $MgCl_2$, and proprietary additives in a buffer optimized for PCR reaction), sterile water (3 µl $reaction^{-1}$), forward and reverse primers (0.5 µl $reaction^{-1}$) (Table 2). After reconfirmation of annealing temperatures, the DNA from germplasm lines was subjected to PCR using SSR markers for PCR amplification. The PCR amplification was carried out in a Thermal Cycler (Master cycler gradient, Eppendorf, Hamburg, Germany). The amplification cycles and PCR have one cycle of initial denaturation at 95°C for 5 minutes followed by 35 to 45 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 45 seconds which vary with different markers (Table 2) and extension at 72°C for 1 minute. After these cycles, final extension will be carried out at 72°C for 8 minute and hold it at 4°C until removal of sample.

2.3. Microsatellite marker analysis

Four SSR primer pairs (VM1, VM3, VM31, AG1/ AF48383) which were found to be linked to CPMV resistance in cowpea (Gioi et al., 2011) and Four SSR primer pairs (M15, M135, M80 and Y96) which were found to be linked to BCMV resistance in cowpea (Manjunatha et al., 2017) were used to detect polymorphism among the cowpea germplasm for resistance and susceptibility to the said diseases. Genotypic data was subjected to single marker analysis which is carried out using generalized linear model (GLM) procedure of statistical analysis system (SAS) to find out the association of markers with root traits and R^2 values were worked out to find the amount of variability explained by the markers.

2.4. Statistical analysis

The amplified gel pictures obtained from eight markers were scored using binary codes. The presence of a band was scored as 1 and absence as 0. The binary data generated for all the genotypes for the polymorphic markers was entered in the NT edit program of NTSYpc version 2.02 software. The similarity matrix was used to generate dendrogram using the SHAN module for cluster analysis. A closely related diversity measure is the polymorphic information content (PIC). The summary statistics including the number of alleles per locus, major allele frequency, gene diversity and polymorphic information content (PIC) values were determined using power maker version 3.23, a genetic analysis software. The PIC values for each SSR are estimated by determining the frequency of alleles per locus using the following formula:

$$PIC=1-\sum_{i=1}^{n} P_i^2-\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

Where, P_i and P_j are the population frequency of the i^{th} and j^{th} allele, calculated for each SSR marker and summation extends over n alleles detected for the i^{th} marker.

Pairwise genetic similarity (GS) was calculated among the cowpea accessions along with check varieties using Jaccard's

Table 2: SSR marker used with annealing temperature and expected amplicon size

Sl. No.	Markers		Sequence 5' → 3'	Annealing temp (°C)	Anticipated size bp)
1.	MA-15	F	AGTTTGGTCCAGGAGTGCAAC	56	379
		R	GACATGACGAGGAGGCACTTT		
2.	MA-80	F	GTTTGATCCTACCTGGTGCCAT	60	370
		R	GCTCATGATTCAAGTCAAGGAC		
3.	Y-96	F	CACTGTGATGCTTTCTGTCAATTG	52	312
		R	CCCCAGTATGCATCCAA		
4.	M-135	F	AAAAGGTTTTTGGGGTGGA	54	250
		R	CCCCGTATGCATCCAAC		
5.	VM-1	F	CACCCGTGATTGCTTGTTG	58	135
		R	GTCCCCTCCCTCCCACTG		
6.	VM-3	F	GAGCCGGGTTCAATAGGTA	58	171
		R	GAGCCAGGGCAGAGGTAGT		
7.	VM-31	F	CGCTCTTCGTTGATGGTTATG	56	200
		R	GAAAAAGGGAGGAACAAGCACAAC		
8.	AG1/AF48383	F	CATGCAGAGGAAGCAGAGTG	58	132
		R	GAGCGTCGTCGTTTCGAT		

similarity coefficient (Jaccard, 1908). Which is given by j=N11/(N11+N10+N01), (where N11 is the number of bands present in both individuals; N10 is the number of bands present only in the individual i; N01 is the number of bands present only in the individual j; and N represent the total number of bands). The values of GS may range from '1' (identical profiles for all marker in the two varieties) to '0' (no common bands).

3. RESULTS AND DISCUSSION

3.1. Screening cowpea genotypes under field condition for yellow mosaic disease (YMD)

To identify the resistance in cowpea genotypes a total of 120 genotypes were screened under field conditions during *kharif,* 2020. The percent disease incidence was recorded. The percent of disease varied from 0 to 33.33% and the highest disease incidence was recorded in IC-202777 and IC-237422 (33.33%). Among the 120 genotypes screened under field condition, 28 genotypes showed an immune reaction, 15 genotypes showed resistant reaction, 52 genotypes showed a moderately resistant reaction, 14 showed moderately susceptible, 11 genotypes showed a susceptible reaction. Results of the genotypes reaction for disease are presented in table 3, and these genotypes were also used for screening of disease using SSR disease resistant specific markers. Single marker analysis was carried out for yellow mosaic disease based on the phenotypic disease scoring data done on the field and the genotypic marker scoring data.

3.2. Validation of SSR markers linked to the cowpea mosaic virus (CPMV) disease resistance in cowpea

To detect polymorphism between resistant and susceptible cowpea genotypes, genomic DNA of resistant and susceptible genotypes was initially used as a template for PCR amplification using disease specific SSR markers. It was confirmed that SSR markers could distinguish resistant and susceptible genotypes in cowpea with different DNA fragment sizes.

Four SSR markers linked to CPMV resistance in cowpea *viz.,* VM1, VM3, VM31, and AG1/AF48383 are used for validation. Among these markers, only VM1 and AG1/AF48383 showed a polymorphic band between susceptible ones on the 3% agarose gel (Figure 1), and the failure of other two SSR marker amplification could be due to allele specificity to the parental genotypes in which they are not identified or may not be very tightly linked to the gene of interest (Mondal et al., 2012). The detailed information of passed two markers such as their polymorphic alleles, major allelic frequency, genetic diversity, and the polymorphism information content (PIC) is listed in table 4. An average of two polymorphic alleles was obtained. The PIC varied from 0.13 to 0.51 with an average of 0.32. The PIC value revealed that polymorphism found to be higher for VM1 marker and lower for AG1/AF48383. Single marker analysis

Table 3: Grouping of cowpea genotypes based on their reaction against yellow mosaic disease infection under field condition

Scale	Description	Reaction	No. of genotypes	Genotypes
0	No plants showing disease Symptoms	Immune	28	IC-20280483, EC-394779, NBC-19, EC-458418, NBC-41, IC-402104, IC-25105, EC-458473, EC-472250, IC-4506, EC-458438, IC-402125, NBC-12, IC-402101, CB-10, NBC-39, NBC-21, IC-4506, NBC-7, GWHOPE, IC-202290, IC-402159, GC-3, NBC-68, NBC-6, IC-1071, EC-402159, C-720
1	Plants Showing disease symptoms of>1-5%	Resistant	15	EC-458469, EC-458440, V-16, NBC-44, MBC-25, IC-402090, IC-402172, IC-20285497, IC-2591054, EC-458480, EC-17058409, EC-394708, IC-58905, NBC-18, TOME-774
2	Plants Showing disease symptoms of >5-15%	Moderately resistant	52	IC-462090, IC-422174, NBC-8, IC-9776710, IC -402180, NBC-21, IC-402182, IC-402135, IC-1061, IC-402162, IC-458430, IC-249593, EC-1705849, C-33, IC-402175, IC-402166, EC-458485, NBC-98, GP-86, NBC-7, GENOTYPE-36, IC-219489, EC-458402, EC-170604, NBC-30, EC-472257, IT-9749938, GC-810,EC-458483, IC-402106, IC-249141, NBC-51, NBC-43, EC-102065, EC-458417, GP-132, NBC-38, KM-5, EC-458418, NBC-36, NBC-14, V-604293, C-325, IT-971549938, NBC-42, GP-154, EC-458470, GP-161, C-457, EC-458425, IC-402048, NBC-40
3	Plants Showing disease symptoms of >15-25%	Moderately susceptible	14	IC-402154, IC -402164, CDP-15, EC-394779, CP-98, EC-472252, V-240, EC-075180, IC-249141, EC-458442, IC-1070, IC-402180, EC-458489, EC-492292
4	Plants Showing disease symptoms of 25-50%	Susceptible	11	C-152, IC-202777, NBC-16, IC-402158, EC-472250, EC-472267, IC-237422, KBC-2, NBC-29, NBC-32, NBC-33
5	Plants showing disease symptoms of >50%	Highly susceptible	0	-Nil-

Table 4: Characteristics of polymorphic cowpea SSR markers linked to CPMV resistance

SSR marker	No. of alleles	Major allele frequency	Genetic diversity	PIC*
VM1	2.00	0.60	0.56	0.51
AG1/AF48383	2.00	0.85	0.19	0.13
Mean	2.00	0.77	0.37	0.32

* PIC- Polymorphic information content

(SMA) revealed that, out of two SSR markers, AG1/AF48383 marker explains 3.80% of phenotypic variation which is superior to VM1 with phenotypic variation of 3.56% thus revealing among these two markers, AG1/AF48383 marker could be used in marker assisted breeding to identify resistant gene against CPMV disease. The marker trait association for Cowpea mosaic virus disease resistance are shown in table 5.

3.3. Validation of SSR markers linked to the Bean common mosaic virus (BCMV) resistance in cowpea

Among 4 SSR markers linked to BCMV resistance in cowpea, *viz*., MA15, MA80, M135, and Y96 only

Table 5: Marker-trait association for CPMV disease resistance in cowpea.

Source of variation	Degrees of	VM1		AG1/AF48383	
		Mean sum of squares	R^2	Mean sum of squares	R^2
Between marker class	1	88.30	3.56*	38.63	3.80**
Within marker class	118	78.17		78.59	

* Significant at p=0.05, ** Significant at p=0.01; Note: R^2 Marker shows the percent phenotypic variation explained by the locus under different analytical model

M135 did not reported to be show amplification while all the other markers showed polymorphic band between susceptible in the 3% agarose gel (Figure 2). The detailed information of these three markers such as polymorphic alleles, major allelic frequency, genetic diversity, and the

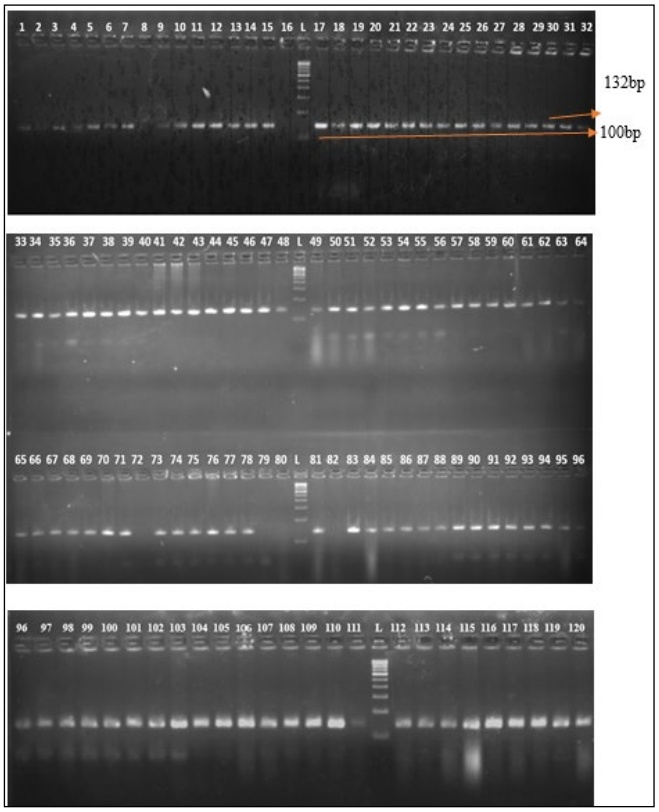

Figure 1: Electrophoresis pattern of PCR amplified fragments of cowpea genotypes with SSR markers (AG1/AF48383). L: 100bp DNA ladder; Lane 1-120: cowpea genotypes; Note: 132bp for CPMV resistance

polymorphism information content (PIC) is listed in table 6. The PIC varied from 0.24 to 0.36 with an average of 0.28, which revealed that among 3 markers MA80 with PIC value 0.36 showed intermediate polymorphism and other two markers MA15 and Y96 with PIC value 0.24, showed low polymorphism. Single marker analysis (SMA) revealed that out of four SSR markers, the marker MA15 explains 3.96% of phenotypic variation. This showed that among these three markers MA15 marker could be used in marker assisted breeding to identify resistant genotypes against BCMV disease. Similar results were reported by Manjunatha et al. (2017) where 10 SSR markers were used to screen the cowpea genotypes against BCMV resistance. The markers trait association for *Bean common mosaic virus* disease resistance are shown in table 7.

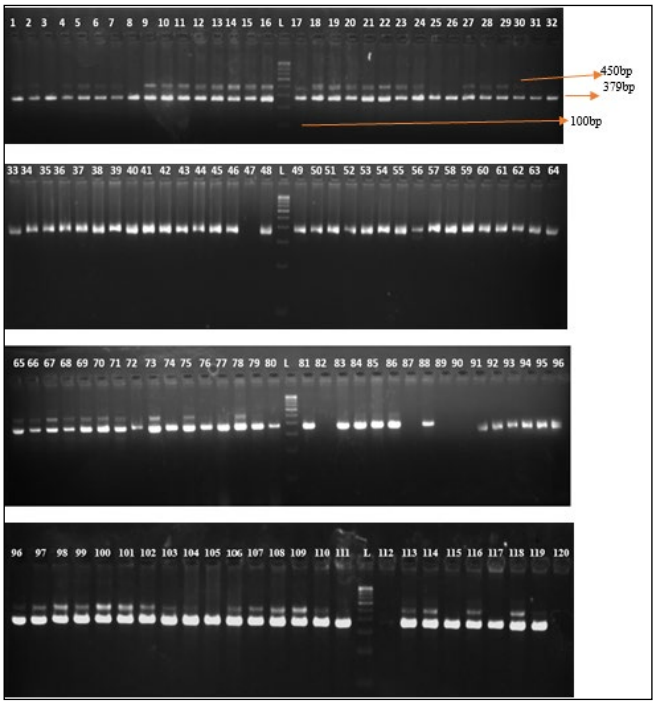

Figure 2: Electrophoresis pattern of PCR amplified fragments of cowpea genotypes with SSR markers (MA-15). L: 100bp DNA ladder; Lane 1-120: cowpea genotypes; Note: 379bp for BCMV resistance

Table 6: Characteristics of polymorphic cowpea SSR markers linked to BCMV resistance

SSR marker	No. of alleles	Major allele frequency	Genetic diversity	PIC*
MA80	2.00	0.53	0.48	0.36
MA15	2.00	0.82	0.27	0.24
Y96	2.00	0.84	0.28	0.24
Mean	2.00	0.73	0.34	0.28

* PIC- Polymorphic information content

3.4. Variability analysis

A dendrogram of 43 cowpea immune and resistant genotypes was constructed by the UPGMA analysis based on the genetic similarity (Jaccard's coefficient) for CPMV and BCMV. The UPGMA analysis done for BCMV disease was broadly grouped into two major clusters of all the 43 genotypes. Two major clusters were further divided into

Table 7: Marker-trait association for BCMV disease resistance in cowpea

Source of variation	Degrees of freedom	MA15		MA80		Y96	
		Mean sum of squares	R^2	Mean sum of squares	R^2	Mean sum of squares	R^2
Between marker class	1	19.29	3.96*	147.53	3.60**	176.91	3.87*
Within marker class	118	78.75		77.66		77.42	

*p=0.05; **p=0.01; R^2 Marker shows the per cent phenotypic variation explained by the locus under different analytical mode

two sub-clusters (Sc) at 56% similarity. The 39 genotypes of sub cluster-I separated into two groups with 65% to 70% similarity, whereas, 4 genotypes of sub cluster-II formed two different groups with 70% to 75% similarity. The clustering of genotypes couldn't clearly differentiate resistant and susceptible genotypes. However, immune genotypes *viz*., IC-402125, NBC-7, IC-1071 and EC-402159 grouped in sub-cluster of cluster II (Figure 3).

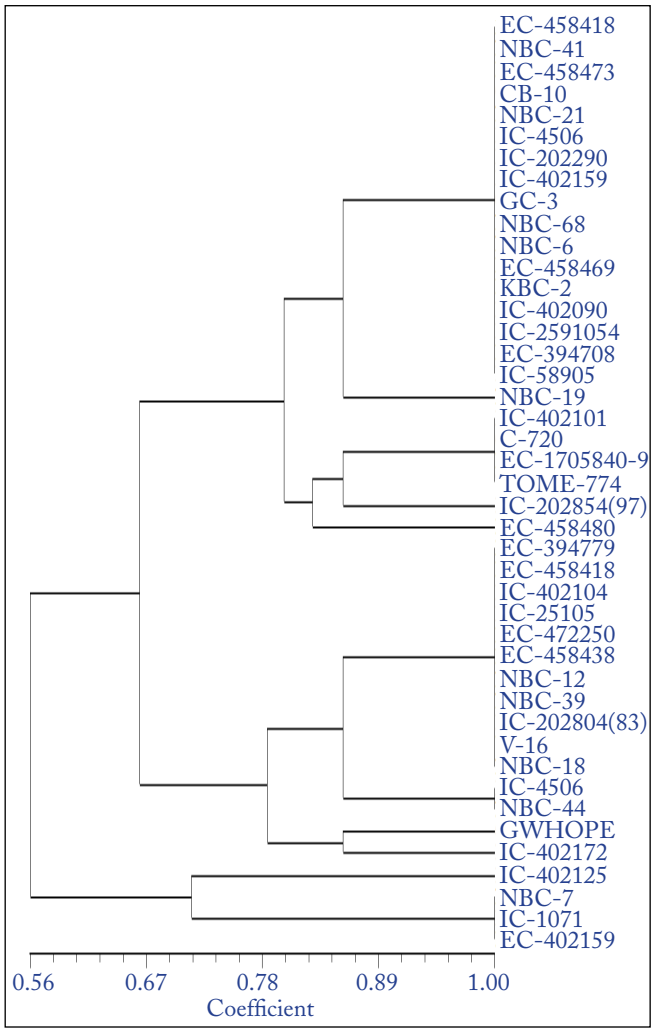

Figure 3: Dendrogram of genetic relationship among cowpea genotypes for BCMV disease based on SSR markers

The UPGMA analysis done for the CPMV disease was grouped into two major clusters of all the 43 genotypes. Two major clusters were further divided into two sub clusters at 54% similarity. The 34 genotypes of Sc-I separated into two groups at 60% to 65% similarity, whereas, 8 genotypes of Sc-II formed two different groups with 65% to 70% similarity. The clustering of immune and resistant genotypes of yellow mosaic for CPMV specific markers was found to be closely associated among the genotypes (Figure 4).

Soybean genotypes were evaluated for cowpea mild

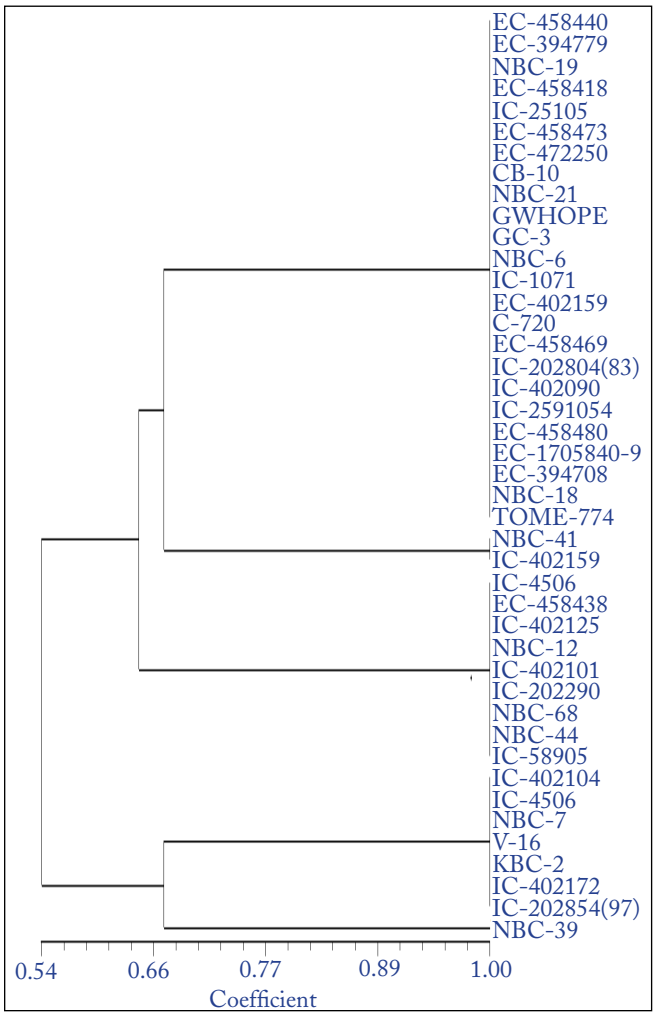

Figure 4: Dendrogram of genetic relationship among cowpea genotypes for CPMV disease based on SSR marker

mottle virus (CPMMV) resistance through phenotypic reaction and genotypic analysis. 500 genotypes were used for screening CPMMV resistance using linked 24 SSR markers. Among which three SSR markers are significantly associated with CPMMV resistance (Yadav et al., 2023). Mungbean genotypes were screened against *mungbean yellow mosaic India virus* (MYMIV) using 256 genome-wide microsatellite markers, among which 93 polymorphic markers were used in association studies. 15 microsatellite markers were reported to link with MYMIV resistance, among them three microsatellites showed 11–14% phenotypic variation (Singh et al., 2020).

Sai et al. (2017) reported the inheritance of yellow mosaic caused by MYMV with monogenic inheritance pattern through SCAR markers and two SCARs namely CM9 and CM15 were found strongly associated with MYMV resistance (Sai et al., 2017). The potentiality of SSR markers in various application in different crops are well demonstrated by number of researchers but still substantial

number of cowpea microsatellites are not available in public domain. EST-derived SSR markers are that once mapped, they will always be associated with the genes carrying them and locus-specificity and transferability across genotypes within the species (Gupta et al., 2012).

4. CONCLUSION

Among four SSR markers used for BCMV resistance, three markers showed desired amplification. Whereas two out of four markers linked to the CYMV resistance showed desired amplification. These markers will be further useful for further validation of other cowpea genotypes against BCMV and CPMV resistance by marker assisted selection.

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