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Screening of important bean genotypes/collections for resistance against Common Bean Mosaic Virus using molecular markers

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Abstract
Bean Common Mosaic Virus (BCMV) causes one of the most serious diseases affecting common bean production. This potyvirus is spread non-persistently by aphids and is also seed-borne. In present investigation out of 132 genotypes selected for screening for BCMV only 8 genotypes (WB-399, WB-640, WB-359 & WB-375, WB-494, WB-933 & WB-939, WB-335) were found phenotypically resistant against it, which was later ascertained by molecular analysis by different microsatellite markers. The variable number of bands produced by different primers ranged from 5 to 22 with an average of 11.4 bands per primer and with level of polymorphism 24.5%. Among the molecular markers used, SSR marker named 835 produced a maximum of 6 alleles, followed by marker 841 then by marker 827 then by BMD-1 followed by ROC-11then followed by marker EIF-4E then followed by SBD-5 and finally by SW-13 that produced 3 alleles each. The polymorphism information content (PIC) was calculated for all the markers, and was highest for Primer BMD-6 which exhibited PIC value of 0.49 and the lowest for the primer BMD-2 (0.22). Hence, primer BMD-6 was observed to be highly informative and serve as an effective and useful marker to determine the genetic differences among the common bean accessions and also to study the phylogenetic relationships.

Keywords: bean, genotypes, resistance, virus, markers

Introduction
In *Phaseolus vulgaris* two main types of symptom occur, depending on virus strain and host genotype: the common mosaic often associated with leaf malformation, and 'black root' characterized by the systemic necrosis and plant death [1]. This virus causes mosaic in most other susceptible *Phaseolus* species [2] [3]. A virus with flexuous filamentous particles *c.* 750 nm long and 12-15 nm wide, containing single-stranded RNA, transmitted by mechanical inoculation, by several aphid species in a non-persistent manner, and through seed and pollen. The virus induces the formation of cylindrical inclusions in the cytoplasm of infected cells. In nature it is mainly restricted to *Phaseolus* species, especially *P. vulgaris,* being found wherever this legume is grown. *Phaseolus vulgaris.* A set of differential varieties possessing the dominant necrosis gene and/or different strain-specific recessive genes is available for strain identification [1]. The virus is transmitted in a non-persistent manner by several aphid species, notably *Acyrthosiphon pisum, Aphis fabae* and *Myzus persicae* [4], [5]. Other reported vector species include *Aphis gossypii, A. medicaginis, Hyalopterus atriplicis, Macrosiphum ambrosiae, M. pisi* and *M. solanifolii* [6]. Most of these aphid species do not colonize *P. vulgaris* but transmit the virus efficiently as winged migrants. Aphids acquire the virus optimally in probes of 15-60 sec and transmit it within 1 min [7],[5],[8].The high incidence of seed transmission is probably the most important factor affecting initial crop infection and the world-wide distribution of the virus. Depending upon the bean genotype and virus strain tested, up to 83% of the seed produced by infected plants may give rise to common mosaic-affected plants [9], [10], [11]. The virus is not appreciably affected during prolonged storage of the seed up to 30 years [12]. The virus is located mostly in the embryo [13]; virus in the seed coat is inactivated during seed maturation [14]. [15]. Classifies the virus in Subdivision I of the potyvirus group, according to the morphology of the cylindrical (pinwheel) inclusions induced in the cytoplasm of infected cells.

Materials and methods
Plant material
The material for the present study comprised of 132 germplasm accessions of common bean (both local and exotic). The pedigree of the lines used is given in Annexure-I. The material

represented diverse market classes of common bean based on growth habit, seed shape, colour and pod characters. The present investigation was undertaken during 2014-2015 at Centre for Plant Biotechnology, SKUAST-K, Shalimar.

Screening of genotypes for identification of resistant genotypes for BCMV

The screening of genotypes was done by artificial inoculation of genotypes as described by [16].

Estimation of genetic diversity in bean lines using microsatellite (SSR) markers

Genomic DNA extraction

Plant DNA was isolated using CTAB (Cetyl Trim ethyl Ammonium Bromide) method as modified by [17].

Selection of primers

The primer markers utilized in the present study were selected on the basis of literature available. Among these, a set of 13 microsatellite markers were selected.

PCR amplification

In vitro amplification using polymerase chain reaction (PCR) were performed in a 96 well microtiter plate in a Bio-Rad or Eppendorf master cycler using 75 ng of genomic DNA of each genotype in a final volume of 20 µl per reaction. The stock and final concentration of different components that were used in PCR is given in table 1& 2.

Visualization of PCR products and Scoring of primer allele profile

The primer allele sizes were determined by the position of bands relative to the DNA ladder. Total number of alleles were recorded for each microsatellite marker in all the genotypes under study by giving the number to amplified alleles as 1, 2, 3, 4 and so on. Number 1 was given to the allele having highest molecular weight. The amplified bands were recorded as 1 (band present) and 0 (band absent) in a binary matrix. The accessions that did not show any amplification were scored as missing values if amplification were not repeated and as null alleles if the amplification was repeated 2-3 times. If the band appeared in the negative control the whole PCR reaction experiment were discarded and repeated again.

Table 1: Stock and final concentration of different components used in PCR

Components	Stock Conc	Volume (µl)	Final Conc
Water	-	3.8	-
PCR buffer	10X*	2.0	1X
$MgCl_2$	25mM	1.2	1.5mM
dNTPs	1mM	4.0	100µM
Primer Forward	5µM	1.0	0.25µM
Primer Reverse	5µM	1.0	0.25µM
Taq Polymerase	5U/µl	0.2	1Unit
DNA template	15ng/µl	5	75ng
Total		20	

*10X PCR buffer: 10mM TrisHCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01 % Gelatin.

The reaction mixture was overlaid with a drop of low molecular weight mineral oil (SIGMA) and placed in 96 well thermal cycler. Amplification were performed using temperature profile mentioned in Table 1.

Table 2: Temperature profile used in PCR

Step	Temperature	Time (minutes)	No. of cycles
Initial denaturation	94	4	1
Denaturation	94	1	35
Annealing	50-60	2	
Elongation	72	2	
Final Extension	72	7	1
Hold	12		

Polymorphism information content (PIC) values and effective multiplex ratio (EMR) and marker index (MI)

The PIC values as described by [18] were used to refer to the relative value of each marker with respect to the amount of polymorphism exhibited. PIC values for each of the primer were estimated using formula given by [19].

$$PIC = 1- \sum_{i=1}^{n} (P_{ij})^2$$

Where P_{ij} is the frequency of j^{th} allele in i^{th} primer and summation extends over 'n' patterns. PIC is synonymous with the term 'gene diversity' as described by [20]. The PIC takes into account not only the number of alleles that are expressed but also the relative frequencies of those alleles [21].

Number of loci (L): number of loci in case of RAPD is equal to the total number of bands ($n_p + nn_p$) obtained. Number of loci per assay unit: nu = L/U. Fraction of polymorphic loci $\beta = n_p/n_p + nn_p$. Effective multiplex ratio E = nuβ. Marker index MI = PIC × β × α. [22].

Experimental findings

The present investigation entitled "Identification and morpho-molecular characterization of resistant bean common mosaic virus (BCMV) genotypes of common bean (*Phaseolus vulgaris* L.)" was undertaken to generate the information on No. of genotypes found resistant against BCMV from 132 genotypes using two susceptible checks *viz.*, SR-1 and WB-967. Genetic divergence was observed on the basis of morphological data in 10 common beans genotypes. Besides it, molecular characterization of resistant bean common mosaic virus genotypes of common bean (*Phaseolus vulgaris* L.) was undertaken to elicit information on genetic polymorphism, similarity matrix, polymorphic information content, and cluster analysis.

Screening

Out of 132 genotypes, using two known susceptible checks *viz.*, SR-1 and WB-967, only 8 genotypes (WB-399, WB-640, WB-359 & WB-375, WB-494, WB-933 & WB-939, WB-335) were found resistant against BCMV, which were then objected to calculate molecular data (Fig 1 & 2; Table 3& 4).

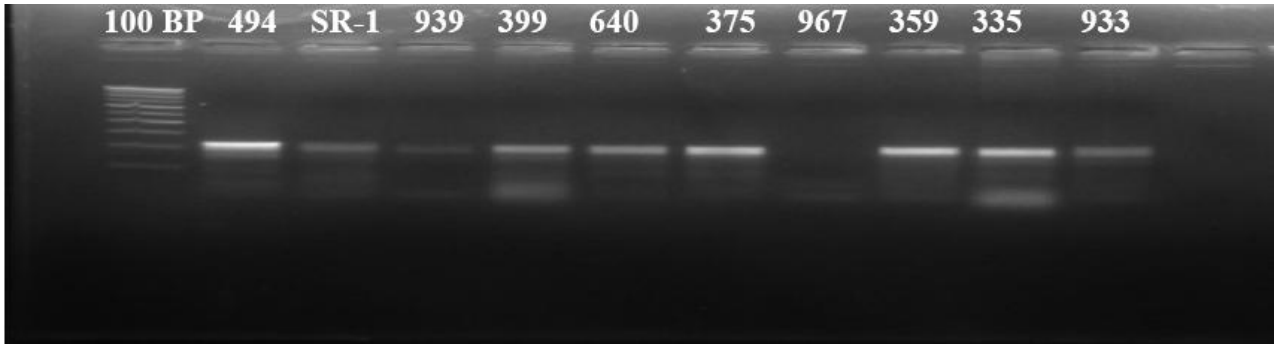

Fig 1: Ethidium bromide stained gel picture showing genetic relationship among 10 common bean (*Phaseolus vulgaris* L.) genotypes using primer BMD-3

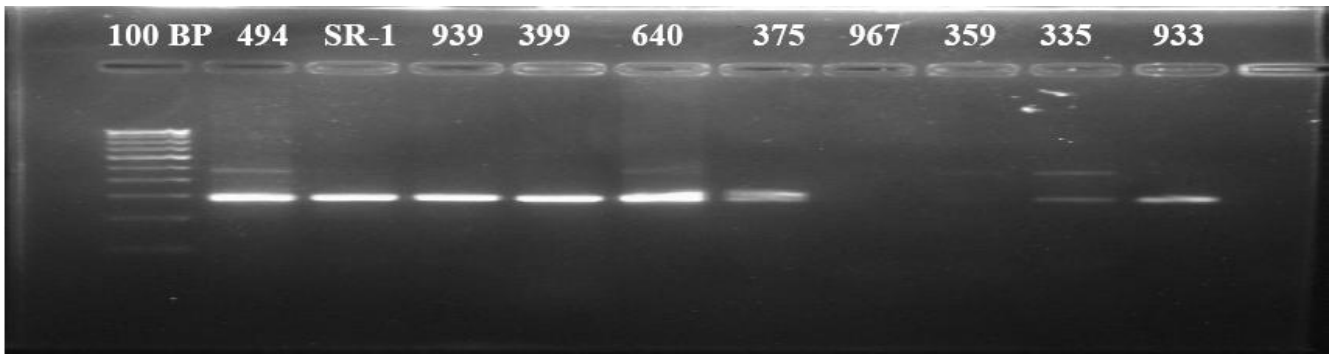

Fig 2: Ethidium bromide stained gel picture showing genetic relationship among 10 common bean (*Phaseolus vulgaris* L.) genotypes using primer ROC-11

Table 3: Percentage of polymorphism obtained by PCR amplification of DNA in 10 common beans (*Phaseolus vulgaris* L.) genotypes

Primer	Total No. of bands	No. of polymorphic bands	Percentage of polymorphism
841	13	3	23
835	17	8	47
827	5	2	40
BMD-3	9	0	-
BMD-2	8	0	-
BMD-1	12	3	25
BMD-6	6	0	-
BMD-4	16	6	37.5
ROC-11	13	4	30.7
Eif-4e	9	0	-
SG6	8	0	-
SBD-5	22	13	59
SW-13	10	1	10
Total	148	40	
Mean per primer	11.4	3.07	

Table 4: Polymorphic information content (PIC) of 13 primers across 10 genotypes of common bean (*Phaseolus vulgaris* L.)

Primer	Polymorphic information content (PIC)	Effective multiplex ratio (EMR)	Marker index (MI)
841	0.44	0.09	0.039
835	0.32	0.08	0.025
827	0.29	0.06	0.017
BMD-3	0.34	0	0
BMD-2	0.22	0	0
BMD-1	0.28	0.12	0.033
BMD-6	0.49	0	0
BMD-4	0.24	0.30	0.072
ROC-11	0.30	0.13	0.039
EIF-4E	0.38	0	0
SG6	0.38	0	0
SBD-5	0.26	0.43	0.111
SW-13	0.44	0.03	0.013
MEAN	0.39	0.09	0.035
CD (0.05%)	0.04	-	-

Discussion

The morphological analysis revealed that BCMV is present in Kashmir valley with high incidence (Fig 1&2, Table 3& 4). Out of 132 genotypes only 8 were found to be resistant against BCMV [16]. Similarly, based on various morphological characteristics, various brinjal genotypes/collections were observed to exhibit variable responses to pest (*Leucinodes orbonalis* Guenee) [23]. In the present study, microsatellite markers were used to determine the extent of diversity among 10 genotypes of common bean. This findings can have implications for detailed studies on genetic diversity among genotypes [24]. Further, the resistance breeding based on molecular approaches intergraded with conventional one help in green revolution in food security [25], [26]. The Molecular markers such as SSR can be used to study genetic diversity among genotypes [27], [28], [29]. Thirteen primers amplified in 10 genotypes produced 37 alleles which were used to generate marker profiles. The number of bands produced by different primers ranged varied from 5 to 22 with an average of 11.4 bands per primer and level of polymorphism 24.5%. Among the primers used, 835 produced a maximum of 6 alleles, followed by 841, 827, BMD-1, ROC-11, EIF-4E, SBD-5 and SW-13 produced 3 alleles each. [30]. Found that from 20 SSR markers evaluated using 85 accessions, the number of alleles per locus ranged from 3 to 10 with a mean of 7. They also recorded a lower observed heterozygosity (He) of 0.026 compared to the expected heterozygosity (He) of 0.622, suggesting that it is also an inbreeding crop. Further, the findings of higher polymorphism detected by SSRs are in agreement with earlier findings of [31], [32], [33] and [34]. In contrast, [35] reported an average of 11 alleles per locus in an SSR analysis of a worldwide common bean collection, whereas [36] reported over 72 alleles in an SSR analysis, with an average of 18 alleles per locus, in an international collection of common beans from Andean and Mesoamerican gene pools. SSRs are characterized by their hyper variability, abundance, reproducibility, Mendelian mode of inheritance and co-dominant nature [37]. Amplification success and polymorphism declines with increased genetic distance. The high level of polymorphism of microsatellite markers and their wide cross-species transferability make these new markers useful for mapping and molecular characterization of *Phaseolus* species [37].

Polymorphism information content (PIC) value of each microsatellite marker is a measure of marker diversity. PIC value provides an estimate of discriminatory power of a locus by taking into account not only the number of alleles expressed, but also the relative frequency of those alleles. The polymorphism information content (PIC) was calculated for all the markers. PIC was highest for Primer BMD-6 exhibited PIC value of 0.49 and the lowest for the primer BMD-2 (0.22). Hence, primer BMD-6 was observed to be highly informative in the present study. This indicated that the Primer BMD-6 might serve as an effective and useful marker to determine the genetic differences among the common bean accessions and to study the phylogenetic relationships. In previous report for 35 North American Soybean Ancestors (NASA), five AFLP primer-pairs produced 90 polymorphic (27%) and 242 monomorphic AFLP fragments. The polymorphic information content (PIC) scores ranged from 0 to 0.49. The polymorphism observed in SSR markers among the common bean genotypes in the present study demonstrated the effectiveness of this method in determining genetic variation. All the SSR markers used were found to be highly informative in revealing the genetic diversity analysis. The result of the present study are in accordance with the earlier reports of [38, 34, 39, 40].

Summary and conclusion

The present study revealed that BCMV is present in Kashmir valley of India in high persistence as morphological study was affirmed by molecular analysis.

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