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Evaluation of black gram (*Vigna mungo*) genepool against *Callosobruchus maculatus* and diversity analysis *inter se*

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ABSTRACT

The present study was carried out at ICAR-National Bureau of Plant Genetic Resources, New Delhi during 2020–21 to evaluate the differential reaction of 69 germplasm accessions representing black gram [*Vigna mungo* (L.) Hepper] landraces and its crop wild relatives for resistance against *Callosobruchus maculatus* (Fab.) under artificial infestation set-up using 'No-choice test' method and analyze their genetic diversity using SSR markers. After emergence of adult beetles, the accessions were studied for the growth parameters like total oviposition, exit holes, adult emergence (AE), per cent seed weight loss (PSWL) and growth index (GI), which varied significantly. Based on the key traits, viz. AE and PSWL, the accessions were categorized into six groups, viz. immune (I), resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S) and highly susceptible (HS). Accessions IC259504 (*Vigna vexillata*) and IC424616 (*Vigna mungo*) were immune and resistant to bruchid infestation respectively. Moreover, the genetic diversity parameters such as allele number, PIC values and observed heterozygosity indicated considerable diversity among the accessions. The reported immune and resistant accessions could be used as donor parents in the *Vigna* breeding programme for transferring bruchid resistance factor(s) to agronomically superior cultivars.

Keywords: Black gram, *Callosobruchus maculatus*, Growth parameters, Resistance

Black gram [*Vigna mungo* (L.) Hepper], a promising pulse crop, forms a major part of the vegetarian Indian diet (Indhu *et al.* 2018). However, the production and storage are significantly hampered by substantial on-field and post-harvest damage from bruchids, especially *Callosobruchus maculatus* (Fab.), a field-to-store pest which inflicts maximum qualitative and quantitative damage to black gram seeds during storage (Panigrahi *et al*. 2021). More than 50% loss in seed weight and protein content has been reported in black gram due to bruchid infestation during storage thus rendering the infested grains unsuitable for human consumption (Gujar and Yadav 1978).

The availability of natural sources of immunity or resistance to *C. maculatus* infestation is scarce in cultivated black gram (Duraimurugan *et al.* 2014, Tripathy 2016), which necessitates screening of large and diverse set of germplasm. However, there have been limited efforts to screen black gram germplasm against bruchids resulting in few breeding programmes for introgression of bruchid resistance genes. A possible reason for this may be the narrow genetic base among local cultivars (Pyngrope *et al.* 2015, Suvan *et al.* 2020). Thus widening the available genetic base holds the key to increasing seed yield in black gram which will facilitate the incorporation of bruchid resistance genes for which assessment of genetic diversity forms a vital constituent (Zhang *et al.* 2017, Pratap *et al.* 2021). SSR is a robust tool for molecular characterisation in crops due to its co-dominance and multi-allelic nature. SSRs are reliable because they are locus-specific, PCR-based and easy to score marker systems (Kaewwongwal *et al.* 2015).

Hence, the present experiment was taken up to evaluate the differential response of black gram and its crop wild relatives (CWR) germplasm against pulse beetle (*C. maculatus*) and to understand the genetic diversity of the screened accessions employing microsatellite markers.

MATERIALS AND METHODS

Plant material: Present study was carried out at ICAR-National Bureau of Plant Genetic Resources, New Delhi acquiring 55 black gram landraces from the National Genebank, ICAR-NBPGR, New Delhi during 2020–21. Additionally, 10 wild *Vigna* accessions representing the CWRs, viz. *V. radiata* var *sublobata, V. trilobata, V. vexillata* and four checks, viz. KU-6, Mash-114, PU-11-14 and

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IPU-2-43 were also included in the experimental material. Collection sites of the studied accessions has been shown in (Supplementary Fig 1).

Insect bioassay: Insect culture of *C. maculatus* was reared on seeds of black gram variety in a Biological Oxygen Demand (BOD) incubator at Entomology Lab, Division of Plant Quarantine, ICAR-NBPGR, New Delhi, for 4–5 generations before starting the experiment (Relative Humidity: 65±5% and temperature: 28±1°C). The black gram and its CWR accessions were screened during 2020–21 for their reaction to *C. maculatus* using the 'No-choice test method' under artificial seed infestation (Giga 1995). The experimental design followed was a Completely Randomised Design with 5 replications, comprising 20 healthy and well-dried seeds from each accession that were weighed and placed in glass bottles with perforated lids to allow proper air circulation. The adults (male and female) were released into each perforated bottle @two pairs per replication. Insects were removed after 72 h of oviposition. Observations on various growth parameters were noted, which included total oviposition, adult emergence (AE) Per cent, number of exit holes, per cent seed weight loss (PSWL), mean development period (MDP) and growth index (GI). The number of eggs laid on seeds determined the extent of oviposition on each accession. As soon as adults started emerging, observations were recorded after every 24 h and continued till no emergence. Adult emergence and MDP were calculated using formula described by Howe (1971). Similarly, GI and PSWL were determined using formulae given by Jackai and Singh (1988) and Eker *et al.* (2018) respectively.

Statistical Analysis: The growth parameters of bruchid were subjected to statistical variance analysis using the CropStat 7.2 program (IRRI 2007) to determine significant differences among the studied accessions.

DNA isolation: Freshly germinated leaves of germplasm accessions were used for DNA extraction as described by Doyle (1990). The quality of DNA was checked on 0.8% agarose gel followed by the determination of DNA concentration using NanoDrop (Thermo Fisher Scientific, USA)**.** As per NanoDrop reading, a working dilution of 20 ng/µL was prepared for PCR amplification of SSR markers.

SSR loci analysis: PCR was done in a 15 µL volume consisting of 2.0 µL genomic DNA (40 ng), 7.5 µL OnePCR™ Master-mix (GeneDireX, Taiwan), 0.5 µL of each primer (10 nmol) and 4.5 µL MilliQ water. PCR amplification was performed in a thermal cycler (G-Storm UK), maintaining the following PCR program: initial DNA denaturation at 94°C for 5 min, subsequent 34 cycles of denaturation at 94°C for 1 min, standardized annealing temperature for 1 min and extension at 72°C for 1 min followed by a final extension at 72°C for minimum 7 min. The annealing temperature was standardized by varying the temperature. The amplicon of each SSR loci was separated out on 4% metaphor agarose (Lonza USA) gel for 4 h at a supply of 100 V, and DNAmark^{TM} 100 bp Ladder (G Biosciences USA) was used to determine the size of the amplicons.

A total of 43 microsatellite markers were retrieved from Bangar *et al.* (2018) and selected for studying the genetic diversity among the studied accessions (Supplementary Table 1). The selected primers were screened initially for amplification, out of which 30 displayed polymorphism. The amplified PCR products were scored using PyElph 1.4 (Pavel and Vasile 2012). The parameters of genetic diversity, viz. allele number (An), frequency of major allele (Maf), polymorphic information content (PIC), gene diversity (GD), and observed heterozygosity (Ho), for each g-SSR were calculated followed by construction of Phylogenetic neighbour-joining (NJ) tree using Nei's genetic distance method (Nei *et al.* 1983, Liu and Muse 2005). The genetically diverse populations were rebuilt from version 2.3.4 of STRUCTURE (Pritchard *et al.* 2000). The software was run by adjusting the value of K from 2 to 10. An online tool Structure Harvest was used to identify the most probable populations.

RESULTS AND DISCUSSION

Variability in insect growth parameters: Evaluating diverse germplasm accessions, especially landraces and CWRs is essential for ascertaining trait-specific germplasm. Data from screening under an artificial infestation setup revealed significant differences among the accessions against their response to *C. maculatus* in terms of total oviposition, AE, number of exit holes, PSWL and GI (Table 1). The highest variability was observed for GI (62.09%) followed by AE (35.36%). Oviposition depends on host availability and is influenced by various antixenotic traits, including chemical constituents present on the seed coat (Petzold-Maxwell 2011, Tripathi *et al*. 2015, Tripathi *et al*. 2017).

Table 1 Analysis of variance (ANOVA) for growth parameters of *C. maculatus*

Trait	Range	Mean ± SE	CD (0.05)	CV
OP	9.67-56.0 (IC331454-IC436519)	29.05 ± 0.94	9.44	26.72
AE (%)	5.77-89.44 (IC424616-IC553517)	51.34 ± 2.20	15.58	35.39
Exit holes (No.)	1.67-19.33 (IC424616-IC371765, IC394479)	13.88 ± 0.49	4.39	29.43
PSWL (%)	11.29-63.48 (IC424616-IC541046)	42.00 ± 1.28	10.30	25.24
GI	0.49-22.46 (IC424616-IC524639)	7.72 ± 0.58	4.09	62.09
MDP (days)	3.28-13.0 (IC553517-IC140825)	7.92 ± 0.27	2.12	27.87

OP, oviposition (per 20 seeds); AE, adult emergence; PSWL, % seed weight loss; GI, growth index; MDP, mean development period.

The range for the number of eggs laid was 9.67–56.00. The number of exit holes from insect emergence, which was observed to be a better criterion for assessing resistance than oviposition varied from 1.67–19.33. Adult emergence per cent, PSWL and GI are the most reliable yardstick to determine resistance/susceptibility of accession to bruchid infestation (Jackai and Asante 2003). Adult emergence appears to depend on larval survivability and its ability to counteract chemical deterrents present inside the seed (Amusa *et al.* 2018), extending the developmental period. In our study, AE per cent which varied from 5.77– 89.44, was absent in the immune accession (IC259504) and significantly less in the resistant accession (IC424616). The extent of damage inflicted due to larval feeding inside the cotyledon is manifested as PSWL whose range was 11.29–63.48. Similarly, observation for GI was in the range 0.49–22.46.

Categorisation of accessions for their response to C. maculatus: The accessions were screened for their reaction to *C. maculatus* infestation based on growth parameters of bruchid. A scatter plot among three key traits, viz. AE per cent, PSWL and GI showed a linear trend (Supplementary Fig 2). The accessions were classified into six groups, viz. immune (I), resistant (R), moderately resistant (MR), moderately susceptible (MS), susceptible (S) and highly susceptible (HS) based on AE and PSWL (Supplementary Table 2). Immune and resistant group was represented by one accession each (IC259504 and IC424616 respectively) which were validated in subsequent screening.

SSR polymorphism: Thirty, out of 43 selected microsatellite markers displayed polymorphism and substantial genetic variability among the studied accessions (Table 2). A total of 105 alleles were observed which varied from 2 to 10 alleles per marker. The mean number of alleles per locus detected (3.37) was near the findings of Gupta and Gopalakrishna (2009) and Pyngrope *et al.* (2015) but much lower than reported by Kaewwongwal *et al.* (2015) and Suvan *et al.* (2020). It may be due to a small number of black gram accessions involved in the study, especially wild germplasm. The major allele frequency ranged from 0.28 (mgssr172) to 0.97 (VR256), with a mean value of 0.68 per locus. The observed genetic diversity value among the selected microsatellite markers varied from 0.06 (VR256) to 0.79 (mgssr172) with a mean of 0.41 per locus. The observed heterozygosity varied from 0 to 0.95 with a low mean heterozygosity (0.23) which could be ascertained primarily due to autogamous nature of black gram responsible for high homozygosity (Gediya *et al.* 2019). The PIC value varied from 0.06 (VR256) to 0.76 (mgssr172). However, the mean PIC value representing the discriminatory power of a molecular marker (0.35) was in close agreement with Sangiri *et al.* (2007) using SSR marker. Higher the PIC value more diverse the germplasm hinting its suitability for utilization in breeding programmes. In our study, the primer mgssr172 showed the highest PIC value (0.76) and the maximum number of alleles (10), indicating it most preferable among the selected SSR markers for characterising black gram and related *Vigna* species.

Phylogenetic study and population structure analysis: The scored SSR marker data was used to construct the phylogenetic tree, which grouped the studied accessions into 3 major clusters (Fig 1). Cluster 1, the major one, consisted of 65 accessions, including the wild accessions (represented as red dots) and 4 checks (represented as blue dots) where 7 wild accessions (IC331457, IC524639, IC553505, IC553510, IC553516, IC553517 and IC553520) were found to be tightly grouped. Additionally, Cluster II and III were observed to consist of 2 accessions each (IC436952, IC436702; IC436644, IC436518 respectively). Tondonba *et al.* (2018) and Suvan *et al.* (2020) also documented three genetic groups revealed by diversity analysis using SSR markers in black gram. Similarly, the population structure distributed the studied accessions into three genetic populations recording the most predictable

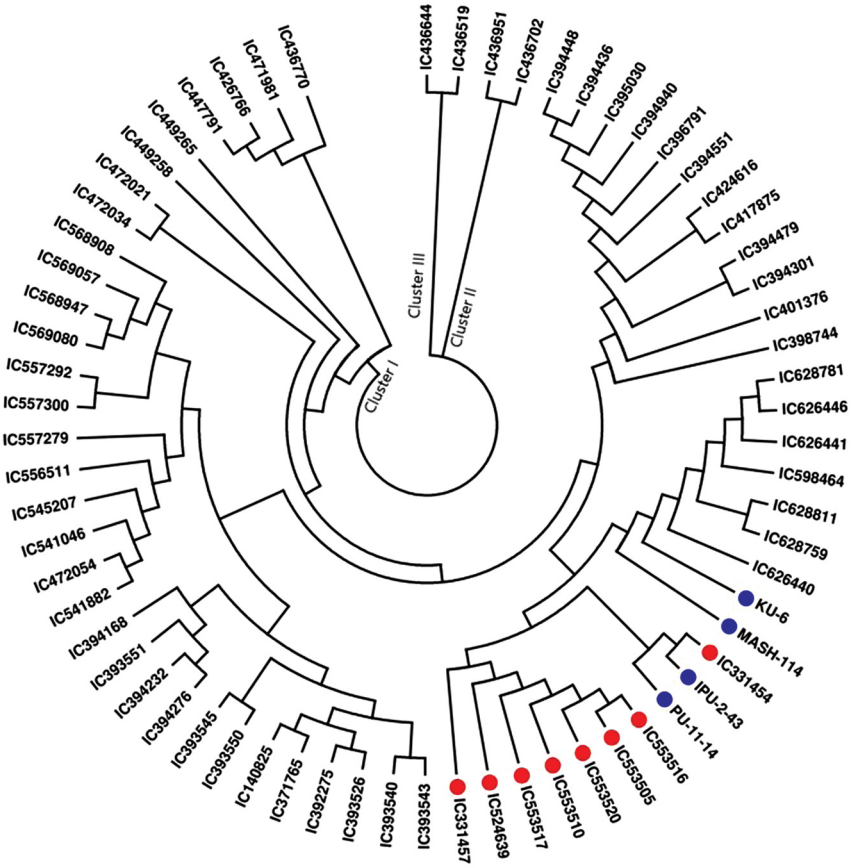

Fig 1 N-J tree of studied accessions using scored data of 30 g-SSRs.

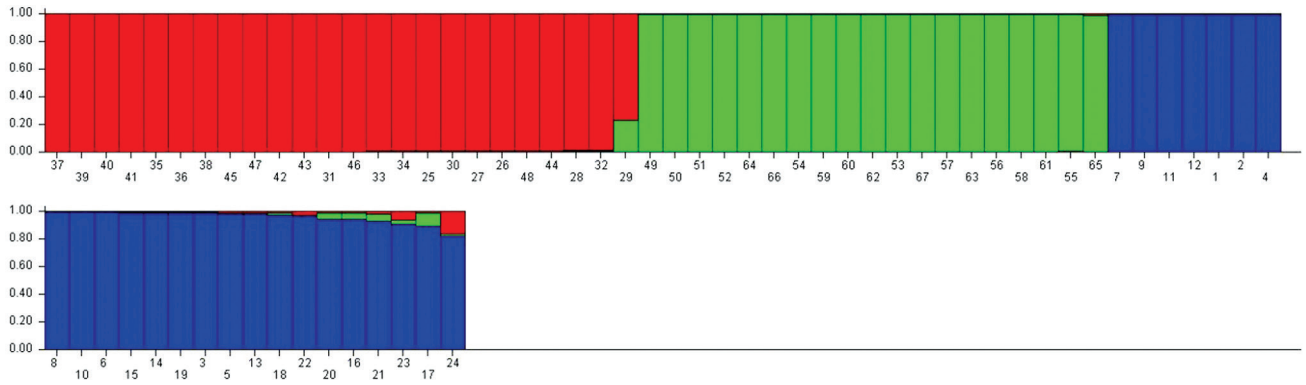

Fig 2 Barplot (K = 3) of population structure of the studied accessions based on 30 g-SSRs.

Table 2 Genetic diversity parameters of g-SSRs

Primer ID	Maf	An	GD	H_o	PIC
VR013	0.7692	2.0000	0.3550	0.0000	0.2920
VR015	0.8281	2.0000	0.2847	0.0000	0.2442
VR016	0.9545	2.0000	0.0868	0.0909	0.0830
VR021	0.6415	2.0000	0.4600	0.0000	0.3542
VR022	0.5085	4.0000	0.5388	0.0847	0.4336
VR024	0.5082	4.0000	0.6009	0.0000	0.5254
VR025	0.5303	2.0000	0.4982	0.0000	0.3741
VR029	0.5690	2.0000	0.4905	0.0000	0.3702
VR032	0.5846	6.0000	0.6187	0.1231	0.5905
VR040	0.7769	5.0000	0.3718	0.3077	0.3421
VR095	0.8881	3.0000	0.2004	0.0149	0.1831
VR102	0.8906	2.0000	0.1948	0.0000	0.1758
VR108	0.4697	4.0000	0.6322	0.3333	0.5633
VR147	0.8955	3.0000	0.1916	0.0000	0.1812
VR256	0.9701	2.0000	0.0579	0.0000	0.0562
VR303	0.9149	4.0000	0.1587	0.0426	0.1515
VR304	0.7500	3.0000	0.3849	0.3696	0.3257
VR338	0.6000	2.0000	0.4800	0.8000	0.3648
VR393	0.5345	4.0000	0.6237	0.0000	0.5685
VM24	0.8478	3.0000	0.2658	0.0217	0.2437
mgssr56	0.9194	3.0000	0.1514	0.1613	0.1457
mgssr142	0.4921	5.0000	0.6099	0.9524	0.5357
mgssr172	0.2857	10.000	0.3585	0.7143	0.7661
mgssr173	0.4717	6.0000	0.6992	0.3396	0.6603
MB122A	0.5522	2.0000	0.4945	0.8955	0.3723
MB738A	0.7692	3.0000	0.3585	0.4615	0.2995
AB128079	0.8983	2.0000	0.1827	0.0000	0.1660
AB128093	0.5263	2.0000	0.4986	0.0351	0.3743
AB100	0.7846	3.0000	0.3569	0.4000	0.3229
BMD-47	0.5755	4.0000	0.5922	0.8491	0.5393
Mean	0.6902	3.3667	0.3933	0.2332	0.3535

Maf, major allele frequency; An, number of alleles; GD, gene diversity; Ho, observed heterozygosity; PIC, polymorphic information loci.

number of subpopulations (K) (Supplementary Fig 3). Bar-plot diagram was used to illustrate the grouping of the genetically pure accessions (Fig 2).

The presented findings reiterate the significance of large-scale characterisation and evaluation of germplasm conserved in the genebanks for the identification of trait-specific genotypes. Besides significant variation in their response to *C. maculatus,* the studied accessions revealed considerable diversity at the molecular level. Further, there is a need to decipher the morphological and biochemical mechanism governing resistance to *C. maculatus* in the identified immune (IC259504) and resistant (IC424616) accessions. Additionally, known bruchid-resistance markers could be employed in unravelling the locus controlling the same in the above accessions.

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