



Mapping of Quantitative Trait Loci for Bruchid Resistance in Blackgram [*Vigna mungo* (L.) Hepper]

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ABSTRACT

Background: Blackgram is one of the most important pulse crops. The post-harvest loss in blackgram is due to *Callosobruchus maculatus* where, the loss can go upto 100 per cent through secondary infestation. Development of cultivar with bruchid resistance can be cost-effective, durable and eco-friendly approach. The present study was conducted to identify markers linked to bruchid resistance in blackgram using the mapping population (RILs) developed from the cross between VBN(Bg) 5 (bruchid susceptible) and *Vigna mungo* var. *silvestris* 22/10 (bruchid resistant).

Methods: A total of 191 RILs were subjected to bruchid screening and the traits viz., days to first adult emergence, mean developmental period, seed damage percent and growth index was observed. To analyse polymorphism between the parents, 343 SSR markers were screened and only 49 (14.29%) SSR markers showed polymorphism between the parents. Bulk segregant analysis (BSA) was done using resistant bulk and susceptible bulk along with parents using polymorphic markers. A total of eight SSR markers were identified as polymorphic in BSA. QTLs were mapped for seed damage per cent and growth index. The flanking markers for the QTLs identified for seed damage per cent and growth index was CEDG088 and CEDG118. Seed damage percent and growth index shared same position. The above markers were validated using four each of resistant and susceptible genotypes of blackgram.

Result: The marker CEDG118 clearly distinguished the resistant and susceptible genotypes. The closely linked SSR marker CEDG118 could be used to screen the germplasm and segregating materials in order to identify bruchid resistant genotypes in blackgram.

Key words: Blackgram, Bruchids, Quantitative trait loci, SSR markers.

INTRODUCTION

Pulses are the major source of dietary protein. Blackgram, a legume crop, has higher protein content (20.8 to 30.5%) and total carbohydrates with a range of 56.5 to 63.7%. Moreover, it contains significant amount of calcium and phosphoric acid. It is also often used to produce fermented foods. Several biotic challenges in the field, storage and abiotic stresses together limit the yields. One of the most significant biotic limitations on the crop has traditionally been storage pests that prey on seeds, which results in significant economic loss during the post-harvest storage period. Among storage pests, bruchid had a negative impact and lessened the crop's economic significance (Somta *et al.*, 2018). Although Blackgram cultivars were shown to be susceptible to pulse bruchid in storage, they were known for their superior performance in the field in terms of pest tolerance. *Callosobruchus* species of bruchids effectively infest blackgram seeds (Bangudu, 1970). The two most important and damaging bruchid species for *Vigna* crops are the cowpea weevil (*Callosobruchus maculatus* L.) and azukibean weevil (*Callosobruchus chinensis* F.). Due to global seed trade, these two bruchid species are widely dispersed throughout almost all continents. Infestation of legume crops by bruchids begins in the field when female bruchids lay eggs on immature pods and larvae eat their way through the pods to the seeds, where they mature into adults by ingesting the nutrients

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from the seeds. Infestation begins in the field and persists throughout storage. After harvest, adult bruchids break out of the seeds and begin a fresh infestation by depositing their eggs right there. This can cause the complete destruction of a seed lot within 2-4 months. Most commonly, chemical fumigation is used to manage bruchids, although it is harmful for human health and not practicable for small-scale farmers and traders. The most practical and sustainable method for managing bruchid infestation is the use of resistant cultivars (Somta *et al.*, 2019). Few resistant varieties with reliable resistant sources needs to be developed and the resistant sources from the wild species needs to be introgressed in the cultivated varieties in order

to overcome post-harvest loss in blackgram (Duraimurugan *et al.*, 2014; Soumia *et al.*, 2015; Somta *et al.*, 2008; Lambirdges and Imrie, 2000). In recent years, marker-assisted breeding has accelerated the breeding programmes for crop improvement in various crops. Several molecular markers have been used for the molecular analysis of grain legumes. In order to develop bruchid resistant lines, understanding the genetic mechanism behind the resistance is essential. To detect effective QTL for bruchid resistance, suitable mapping population is needed. The interspecific hybridization helps to broaden the genetic base of the crop by introgression of genes. Recombinant inbred lines provide sufficient genetic variation for QTL mapping since each line is nearly homozygous (Simon *et al.*, 2008). With this background, the present investigation was undertaken on RIL population to map bruchid resistant genes in blackgram and to identify tightly linked markers for bruchid resistance.

MATERIALS AND METHODS

The experimental material consisted of 191 recombinant inbred lines of F_{10} generation developed from the cross between VBN (Bg) 5 (Bruchid susceptible) and *Vigna mungo* var. *silvestris* 22/10 (Bruchid resistant) through single seed descent method. The experiment was conducted in Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore during 2020 and 2021. The freshly harvested seeds were collected from the field and were stored at -4°C to avoid further infestation. The seeds were used for bruchid (*Callosobruchus maculatus*) screening. The experiment was laid out in completely randomized design and is replicated twice.

Initial stage of screening

The post-harvest damage caused by the bruchids varies from crop to crop depending on the bruchid species and their biotype. *Callosobruchus maculatus* causes up to 100 per cent yield loss in blackgram (Soundarajan *et al.*, 2013) through secondary infestation. The *C. maculatus* was generally differs from other species through two different characters viz., presence of less dense setae on the ventral side of the 2,3,4 abdominal segments (stemites) and presence of serrate type of antenna. The male and female insects of *Callosobruchus maculatus* were identified by general appearance i.e. the colouration on the plate covering the end of the abdomen. In female, the plate is enlarged and darkly coloured on both sides. Initially, a single pair of an adult was cultured in a plastic petriplate containing 50 seeds of greengram and was allowed for oviposition for five days and then adults were removed from the petri plate. After the 20th day, the adults emerged were used in the mass culturing and also for stock culture. Throughout the experiment good aeration for the culturing of bruchids were provided. The stock culture of these adults (*Callosobruchus maculatus*) was maintained on greengram seeds at

constant temperature $32^{\circ}\text{C}\pm 2^{\circ}\text{C}$ and relative humidity of 70 per cent. The freshly emerged adults were used for the screening of RILs against bruchid infestation.

No choice test bioassay method

No choice assay procedure described by Dongre *et al.* (1993) was followed with minor modifications for bruchid screening. Five pairs of newly emerged adults from the stock culture were released on 50 seeds of each line placed in a petri plate with two replications and in each batch susceptible variety CO 5 blackgram was used. The insects were left to remain in petri plates for five days for oviposition. After five days, the adults were removed from the petri plates and periodical observation on adult emergence was done. The damage was calculated on 45 days after inoculation where the susceptible genotype attained 100 per cent seed damage. The observations viz., days taken for first bruchid emergence, mean developmental period (days), seed damage expressed in percentage counted on 45th day of inoculation and growth index was recorded as given by Howe, (1971).

DNA extraction

DNA was extracted from seven days old seedling by CTAB method proposed by Doyle and Doyle, (1987). The quality of DNA was checked by using 0.8% agarose gel electrophoresis using 1X TBE buffer and Ethidium bromide (5 μl /100 ml of Agarose). The DNA samples (2 μl) were mixed with loading dye (3 μl of orange dye) and loaded further into the gel. The electrophoresis was carried out at 80 V for 30 minutes. The gel was documented in BIO-RAD gel documentation system to ascertain the quality of DNA. A total of 343 SSR markers specific to mungbean ((Kumar *et al.*, (2002), Somta *et al.*, (2006), Tangphatsornruang *et al.*, (2009) and Seehalak *et al.*, (2009)), Adzukibean ((Wang *et al.*, (2004), Han *et al.*, (2005), Chaitieng *et al.*, (2006), Wang *et al.*, (2009) and Isemura *et al.*, (2012)), Commonbean ((Blair *et al.*, (2003) and Blair *et al.*, (2010)) and cowpea ((Li *et al.*, (2001)) were employed to study polymorphism between the parents. The PCR reaction mixture composed of 11 μl comprising master mix (7.0 μl), 5 μM primer (2.0 μl) and 50 ng DNA (2.0 μl). The PCR amplification was performed in Bio Rad based on the protocol: initial denaturation at 94°C for 3 minutes, followed by 35 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, final extension at 72°C for 10 minutes and 4°C at Hold. The 10 μl of PCR reaction mixture was subjected to gel electrophoresis in 3 per cent agarose gel and documented using gel documentation unit (Bio- Rad).

Bulk segregant analysis

Bulk segregant analysis (BSA) was carried out according to Michelson *et al.* (1991) and to identify the marker linked with gene of interest. Resistant bulk was formed by mixing an equal amount of DNA from six bruchid resistant lines (seed damage < 10 %) and susceptible bulk was formed

by mixing an equal amount of DNA from six bruchid susceptible lines (100 % seed damage). The markers identified as polymorphic between the parents were used in bulk segregant analysis to identify markers that were polymorphic between the resistant and susceptible bulks. The polymorphic markers from BSA were used for genotyping of all the RILs and finally the markers were scored for segregation pattern for 1:1 goodness of fit using chi square analysis.

QTL analysis and validation of markers linked with bruchid resistance

The genotypic and phenotypic data on days to first adult emergence, mean developmental period, seed damage per cent and growth index were utilized to identify tightly linked markers for bruchid resistant genes using QTLci mapping 4.2. Inclusive composite interval mapping (ICIM-ADD) method was used and markers were grouped based on Logarithm of odds (LOD) score of 3.0. A permutation test was employed to identify the threshold LOD value with a significance level of 0.05. Significant QTL were those with LOD values > 3.0 and permutation test P 0.05. The SER method was used to arrange the markers, using a widow size of 5 as rippling criteria that re-estimates the recombination frequency without affecting the marker's order. The recombination frequency between the markers was converted into centimorgan (cM) distance using Kosambi mapping function (Kosambi, 1944). The highest probability peak of LOD score provides the phenotypic variation (R^2) of QTL. For validation, the flanking markers identified in QTL analysis were screened in different genotypes of blackgram having resistance and susceptibility to bruchid infestation.

RESULTS AND DISCUSSION

Evaluation of RIL population for bruchid resistance

The 191 recombinant inbred lines along with susceptible (VBN (Bg) 5) and resistant (*Vigna mungo* var *silvestris* 22/10) parents exhibited significant difference for bruchid resistance related parameters viz., days to first adult emergence, mean developmental period, seed damage and growth index. The days to first adult emergence of bruchid ranged from 31 to 44 days with mean of 35 days whereas, the average values for mean developmental period, seed damage and growth index was 41 days, 68.58 per cent and 1.68, respectively (Table 1).

Genetic linkage map

Out of 343 SSR markers used in parental polymorphism survey 49 markers (14.29%) identified as polymorphic between the parents. These 49 markers were used in bulk segregant analysis wherein eight markers (16.32%) showed polymorphism between resistant and susceptible bulks. These eight markers were used for the profiling of 191 recombinant inbred lines. Scoring was done and these eight SSR markers showed a goodness of fit test to 1:1

ratio in RIL population by chi square test. The genotypic scoring data was used as input for the linkage map construction. The linkage map covered a total length of 368.73 cM with an average inter- marker distance of 46.09 cM. The maximum interval length of 59.95 cM was observed between markers CEDG198 and CEDG097 and the smallest interval length of 41.08 cM was observed between markers CEDG048 and CEDG088 (Fig 1).

QTL analysis

The genotypic and phenotypic data were utilized to identify tightly linked markers for the bruchid resistant gene. For growth index, one QTL was detected (*Cmgi* 1.1) with a LOD score of 3.64 and was flanked by markers CEDG 088 and CEDG 118. The position of the QTL for growth index was at 360 cM with the marker interval of 56.9 cM. The phenotypic variation explained by the QTL was 8.99 per cent. For seed damage one QTL *Cmsd* 1.1 was detected with an LOD value of 3.78 and flanked by the markers CEDG 088 and CEDG 118 with a position of 360 cM. This QTL explained phenotypic variation of 9.39 percent. QTLs for seed damage and growth index shared the same position (Table 2).

Validation of flanking markers

The flanking markers viz., CEDG 088 and CEDG 118 identified in QTL analysis were validated in a set of bruchid susceptible blackgram genotypes viz., CO 6, VBN 8, VBN 11, CO 7 and bruchid (*C. maculatus*) resistant genotypes viz., TU 80, TU 68 and TU 58. In genotypes CO 6, VBN 8, VBN 11, CO 7, TU 68 and TU 58, the marker CEDG088 produced an approximately 130 bp-sized allele and 120 bp in TU 80. The allele size of the CEDG118 marker was around 180 bp in all susceptible genotypes and 190 bp in all the resistant genotypes viz., TU 80, TU 68 and TU 58 (Table 3). This marker distinguished resistant genotypes from susceptible genotypes with a considerable specificity. The field carry over pest, bruchid (*C. maculatus*) possess major threat to the production as well as storage of blackgram seeds. The bruchid resistance gene was mapped in several legume species viz., mungbean (Kaga *et al.*, 1998; Wang *et al.*, 2016), ricebean (Venkataramana *et al.*, 2016), commonbean (Blair *et al.*, 2010) and adzuki bean (Somta *et al.*, 2018). The susceptibility of blackgram seeds to *C. maculatus* is high when compared to *C. chinensis*. The source of resistance gene for bruchid in blackgram was found in wild progenitor i.e *Vigna mungo* var. *Silvestris* (Fujii *et al.*, 1989; Dongre *et al.*, 1996 and

Table 1: Mean and Range for screening parameters of RIL population against *C. maculatus*.

Traits	Mean	Range
Days to first adult emergence	35	31-44
Mean developmental period	41	37-44
Seed damage on 45 th day (%)	68.58	4-100
Growth index	1.68	0.03-2.64

Kashiwaba *et al.*, 2003). In order to identify genes responsible for bruchid resistance, linkage map followed by QTL study was conducted among 191 recombinant inbred lines developed from the cross VBN (Bg) 5 and *Vigna mungo* var *silvestris* 22/10. The observed traits for bruchid screening expressed significant variation and such a variation was also observed by Kpoviessi *et al.* (2021) in cowpea and Subramaniyan *et al.* (2021) in blackgram. To identify the QTLs controlling the seed damage and growth index with larger phenotypic variance, QTL analysis was carried out using a high-throughput QTL mapping technique viz., bulk segregant analysis which could quickly locate the genomic regions controlling the desired trait by pooling the segregants according to their phenotypes (Michelmore *et al.*, 1991). In order to identify polymorphism between the parents, VBN (Bg) 5 and *Vigna mungo* var. *silvestris* 22/10, 343 SSR markers have been employed and observed 49 markers (14.29 %) as polymorphic and used in bulk segregant analysis. Sarkar *et al.* (2011) analysed only four markers as polymorphic among the parents from a total of 40 SSR markers employed in parental screening (B1 and Sub2) for bruchids in blackgram. Forty nine polymorphic SSR markers in parental polymorphism were used to screen the resistant and susceptible bulks developed for

C. maculatus. A total of eight markers were found to be polymorphic between extreme bulks and also among parents. Seram *et al.* (2021) identified three markers as polymorphic to respective bulks of bruchid screening from the total of nineteen markers surveyed in BSA in greengram. These markers observed to have segregation ratio of 1:1 and this segregation pattern could be used to identify the map position of the gene controlling this trait (Isemura *et al.*, 2010). The linkage map covered a total length of 368.73 cM with an average interval between the markers was 46.09 cM in which length of 783 cM with average distance between the marker of 5.7 cM was observed by Chaitieng *et al.* (2006) in blackgram. The maximum interval of 59.95 cM was observed between markers CEDG198 and CEDG097 and the smallest interval of 41.08 cM was observed between markers CEDG048 and CEDG088. The QTLs *Cmgi 1.1* and *Cmsd 1.1* were identified for growth index and seed damage, respectively flanked by the markers CEDG 088 and CEDG 118. The phenotypic variation explained by the QTLs was 8.99 % for growth index and seed damage was 9.39 %. Phenotypic variation explained by the QTLs was low and was reported to be minor QTL. The low PVE observed may be due to high intervals between the markers and less availability of

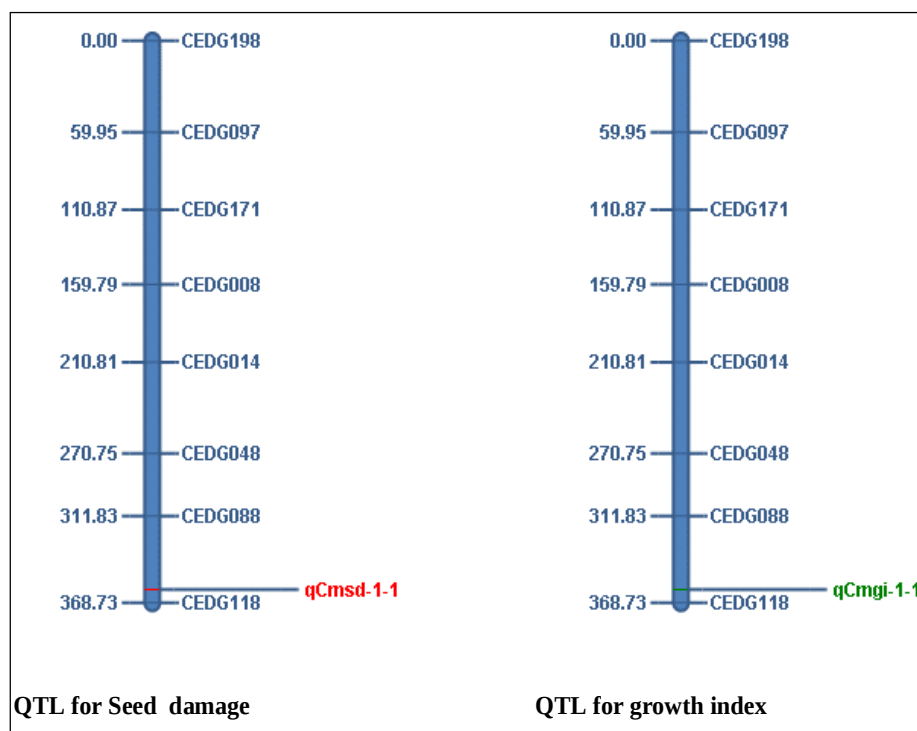


Fig 1: Identification of QTL for bruchid in RIL population of blackgram for seed damage and growth index.

Table 2: QTLs identified for *C. maculatus* resistance in a RIL population.

Trait	QTL name	Position (cM)	Flanking markers	LOD	PVE (%)
Seed damage	<i>Cmsd 1.1</i>	295	CEDG088-CEDG118	3.78	9.39
Growth index	<i>Cmgi 1.1</i>	295	CEDG088-CEDG118	3.64	8.99

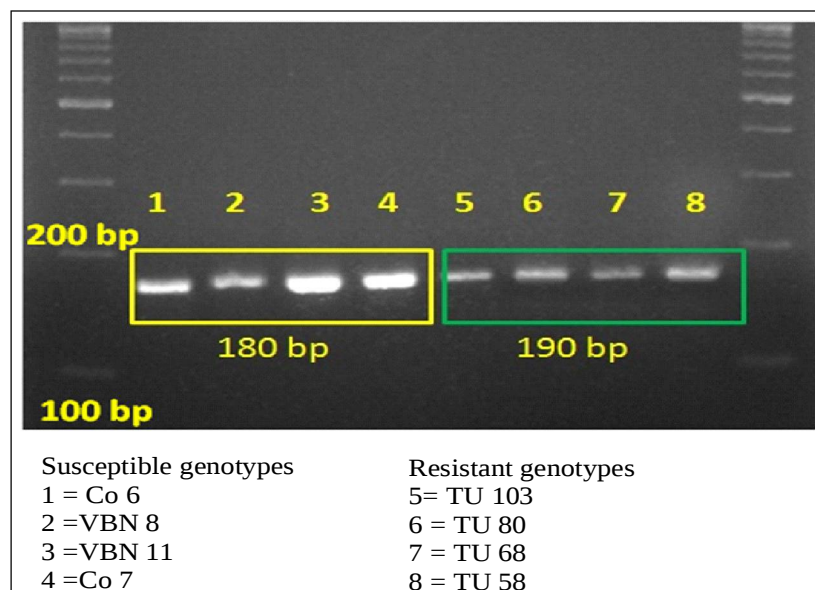


Fig 2: Identification of QTL for bruchid in RIL population of Blackgram for seed damage and growth index.

Table 3: Validation of SSR markers linked with bruchid resistance.

Genotype	Pest response	Allele size in bp (approximate)	
		CEDG088	CEDG118
CO 6	Susceptible	130	180
VBN 8	Susceptible	130	180
VBN 11	Susceptible	130	180
CO 7	Susceptible	130	180
TU 103	Resistant	130	190
TU 80	Resistant	120	190
TU 68	Resistant	130	190
TU 58	Resistant	130	190

polymorphic markers. The population size, number of markers and marker interval play an important role in determining the QTLs, LOD and PVE values. Utz *et al.* (2000) reported low PVE and reported that environmental and genotypic sampling were responsible for low PVE. Li *et al.* (2010) observed that for small population size (~200), the probability of detecting QTL with high PVE is low, no matter how many markers are screened. The flanking markers of the QTL CEDG088 and CEDG118 were validated in each four bruchid susceptible and four resistant (*C. maculatus*) genotypes of blackgram. The marker CEDG 118 produced an approximate allele of 180 bp in all susceptible genotypes viz., CO 6, VBN 8, VBN 11 and CO 7 and 190 bp in all the four resistant genotypes viz., TU 103, TU 80, TU 68 and TU 58 (Fig 2). It clearly differentiated resistant and susceptible genotypes. This reveals the antibiosis resistance mechanism that prevented the insects from feeding the seeds (Misal, 2005 in greengram and blackgram; Lazar *et al.*, 2014, Yao *et al.*, 2015 in greengram; Ahmed *et al.*, 2019, Swamy *et al.*, 2020, Pradhan *et al.*, 2020

in chickpea). Hence, marker CEDG118 was reported be linked to bruchid resistant gene. Souframanien *et al.* (2010) identified CEDG086 and CEDG154 as flanking markers for *C. maculatus* adult emergence and CEDG133 and CEDG 149 as flanking markers for *C. maculatus* developmental period in blackgram.

CONCLUSION

Based on the QTL study and validation, this marker (CEDG 118) could be used in breeding program to identify bruchid resistant lines and to develop bruchid resistant varieties in blackgram. As the marker interval for the identified QTL is high this could be further utilized in the breeding programme after enriching the markers in the identified region of the QTL.

Disclaimers

The authors are responsible for the accuracy and completeness of the information provided in this content.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript

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