https://doi.org/10.48048/tis.2022.3975

Bruchid Resistance Study through Bulked Segregant Analysis: Used as a Preliminary Step for Next-Generation Sequencing

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Received: 28 November 2020, Revised: 5 June 2021, Accepted: 28 June 2021

Abstract

Seed beetles, commonly known as bruchids, are serious insect pests of Indian pulses inflicting high damage during storage. Earlier, a bruchid resistant rice bean landrace (TNAU Red) from Manipur and a susceptible mung bean variety (VRMGg1) from Tamil Nadu were reported and their inter-specific cross was made in Tamil Nadu Agricultural University. In the present study, seeds from the segregating populations (recombinant inbred lines, RILs) of VRM (Gg)1 and TNAU Red were evaluated for bruchid *Callosobruchus maculatus* Fabricius resistance following standard methods under laboratory conditions. Bulked segregant analysis (BSA) is an efficient method for quick identification of molecular markers linked to any specific gene or genomic region. BSA significantly reduces the scale and cost by simplifying the procedure compared to conventional method of analyzing the entire population. BSA was carried out using 3 marker systems with an attempt to identify markers associated with the present trait of interest. Two DNA bulks namely resistant bulk (RB) and susceptible bulk (SB) were grouped by pooling equal amount of DNA from 5 each of resistant and susceptible RILs based on bruchid responses such as seed damage (%), mean developmental days (MDP) and adult emergence from seeds. Marker analysis carried out with 41 polymorphic SSRs showed skewed segregation distortion towards the susceptible parent. However, out of 9 RAPDs and 10 ISSRs, 2 RAPD (OPB08 and OPX04) and 1 ISSR (UBC810) primers amplified an allele at 200 - 300 and 300 - 400 bp, respectively, in resistant parent and resistant bulk but not in susceptible parent and susceptible bulk. The amplification of resistant parent allele in resistant bulk may indicate the presence of bruchid resistant gene(s) in the resistant RILs. This experiment was aimed at verifying the presence of bruchid resistant lines carrying resistant gene(s) prior to further association mapping or QTL mapping studies. Further, such type of study can be applied as a preliminary step in NGS technology.

Keywords: Bruchid resistance, RILs, Bulked segregant analysis, Molecular markers

Introduction

Vigna radiata (L.) R. Wilczek, commonly known as mung bean, is the most economically important pulse species, which is primarily cultivated in South and South-east Asian regions including India. On the other hand, *V. umbellata* (Thunb.) Ohwi and Ohasi (rice bean) is an under-utilized pulse crop, principally produced and consumed in few countries like Japan, China, Thailand, Myanmar [1], North and North-Eastern parts of India [2,3]. Pulses are rich in carbohydrates, vitamins, minerals and digestible low-flatulence proteins which complement Asia's staple rice diet. Being rich in nutrients, pulses are prone to attacks by several diseases and insects before and after harvest. Seed beetles or bruchids are one of the most important storage insect pests attacking both grains and legumes. Bruchids in the genus *Callosobruchus* (mainly *C. maculatus* and *C. chinensis*) of Bruchidae family primarily causes damage in *Vigna* species especially *V. radiata* [4,6] and *V. unguiculata* [7,9]. However, there are reports on bruchid resistance identified in wild progenitor ssp. *sublobata* TC1966 (mung bean) [10], ACC 41 [11], wild

cowpea (TVu2027 line) [12] and rice bean [3,13]. Among these, rice bean (*V. umbellata*), which is one of the under-exploited species, has a very high degree of bruchid resistance and TNAU Red (a landrace) is reported to be highly resistant to both MYMV disease in field [14] and bruchid insect under storage conditions [3].

In India, almost all the commercially available mung bean varieties are more vulnerable to bruchid attack. Attempts have been made to incorporate moderate levels of bruchid resistance from several donor parents into improved mung bean varieties [15] and recently considerable progress are being made in identifying genomic regions and resistance-associated genes in several pulses to different bruchid species [16,17]. Several molecular marker systems have been employed to evaluate DNA polymorphism, to develop genetic linkage maps for various *Vigna* crops and to identify brcuhid resistance associated quantitative trait loci (QTLs). Many workers have developed and used mung bean intra-specific mapping populations for identification of markers linked to this particular trait [18,22]. Moreover, an inter-specific mapping population for marker identification linked with bruchid resistance in *V. umbellata* X *V. nakashimae* [23]. BSA is an effective tool for rapidly recognizing molecular markers that are related to some specific gene or genomic regions. It is a tool for concentrating on regions of interest or sparsely inhabited areas with markers and also for rapidly identifying genes that do not segregate in populations initially used to produce the genetic map [24]. It has been suggested that any polymorphic marker with a clear variation of the 2 extreme bulks will be closely linked to a respective trait of interest or the phenotype [25]. BSA has been carried out to identify markers linked with mung bean yellow mosaic virus (MYMV) resistance [14] in the same segregating populations of VRM (Gg)1 (MYMV susceptible mung bean) X TNAU Red (MYMV resistant rice bean). However, identification of markers in any inter-specific populations [mung bean X rice bean or VRM (Gg)1 X TNAU Red in the present study] using BSA have not been reported as far as bruchid resistance is concerned. In this context, the present experiment was carried out with an aim to confirm resistance phenotyping method and to check for the presence of bruchid resistant lines carrying resistant gene(s) or allele(s) for further association and QTL mapping studies through next-generation sequencing (NGS) technologies.

Materials and methods

Source of insect and seed materials

Bruchid species under study, *Callosobruchus maculatus* (South Indian Thanjavur populations, [3]). were obtained from Indian Institute of Crop Processing Technology (IICPT), Thanjavur and cultured on susceptible mung bean variety "CO - 8" at Insect Pheromone Research Laboratory, Department of Agricultural Entomology, TNAU, Coimbatore, Tamil Nadu, India. For seed materials, a total of 187 recombinant inbred lines (RILs) of a highly bruchid susceptible mung bean variety, VRM (Gg)1 [5] and a completely bruchid resistant rice bean, TNAU Red [3] were obtained from Agricultural Research Station (ARS), Virinjipuram, Vellore, Tamil Nadu. Previously, VRM (Gg) 1 was crossed with TNAU Red [26] and RILs were developed through single seed descent method. The same segregating populations have been used in different studies carried out by several researchers at TNAU [14,27,28]. The populations are presently being maintained at the research station for further generation advancement and molecular breeding studies.

Bruchid resistance evaluation

Seeds from 187 recombinant inbred lines (RILs) of VRM (Gg)1 and TNAU Red were evaluated for bruchid resistance under free-choice test including parental seeds as resistant and susceptible checks. Ten seeds per RIL were used for evaluation under laboratory conditions following standard procedure [10]. Lines showing resistant reaction to *C. maculatus* were again subjected to no-choice test for further resistance confirmation [3,10]. The standard scoring system **Table 1** was followed for resistance categorization based on seed damage per cent [29].

DNA extraction and bulked segregant analysis (molecular marker analysis)

Seeds from each 5 resistant and susceptible lines were sown in pot trays containing coir pith: vermicompost in 1:1 ratio along with the parental seeds for DNA extraction. Genomic DNA was isolated from leaves of 10 day old seedlings (2 - 3 leaved stage) following modified protocol [30]. Extracted DNA was quantified by reading the absorbance at 260 nm using Nanodrop spectrophotometer Nucleic Acid and Protein Quantification instrument, ND - 2,000 (Nanodrop technologies, USA). The crude genomic DNA was run on 0.8 per cent agarose gel stained with ethidium bromide following a standard method for

quality check and was visualized in a gel documentation system (Alpha ImagerTM1, 200, Alpha Innotech Corp, CA, USA).

A total of 60 primers (41 Simple Sequence Repeats, SSR from azuki bean, 9 Random amplified polymorphic DNA, RAPD and 10 Inter Simple Sequence Repeats, ISSR) obtained from Agile technologies, Singapore were used in marker analysis by bulked segregant method. List of primer names and their nucleotide sequences are given (**Table 3-SSRs** and **Table 4-RAPD** and ISSR), respectively. The SSR primers were first screened for positive PCR amplification and polymorphic bands with parental DNA. Only the primers producing polymorphic bands were used for BSA. BSA was performed by pooling DNA from 5 resistant (RIL 19, 53, 158, 165, 169) and susceptible (RIL 60, 66, 116, 121, 123) lines and forming 2 separate DNA bulks, resistant bulk and susceptible bulk, along with the parental DNA. PCR reaction was performed in 15 µL volume of mix containing 2.00 µL extracted DNA (25 ng/µL), 6.00 µL master mix (mixture of dNTPs, Taq polymerase and loading dye), 1.00 µL (10 µm) Primer and 6.00 µL sterile distilled H_2O. The reaction mixture was given a short spin in a vortex machine for thorough mixing of the cocktail components. The PCR reaction was carried out in a DNA thermal cycler programmed to run the following temperature regime: 94 °C for 5 min initial denaturation, 94 °C for 1 min denaturation, 55 °C for 1 min annealing, 72 °C for 1 min extension, 72 °C for 1 min final extension and the temperature profiles 2, 3 and 4 were programmed to run for 35 cycles. Agarose gel (1.5 % agarose for RAPD, ISSR primers and 3 % for SSR) electrophoresis was performed to separate the amplified products. Amplified products were loaded on to the wells along with reference marker (100 bp ladder). Gel electrophoresis voltage was maintained at 80 V for 2 - 3 h. The bands were visualized for the presence of bands and documented in gel documentation system (Alpha ImagerTM1, 200, Alpha Innotech Corp, USA).

Table 3 List of azuki bean polymorphic SSR primers surveyed for BSA.

Sl. No.	SSR locus name	Forward primer (5'-3')	Reverse primer (3'-5')
		Chromosome 1	
1	CEDG090	ATAAGTAGAAATTGGTTCAAATG	GGTTCGTTAAAGTAACTTTTAAT
2	CEDG148	GGGAAGAAGGAAGAAGGAACC	GTGACAAAACACCTGTAGCCATCC
3	CEDG254	CGATGTCTCTTGCTTCAAGG	GTGAAGGACTAGCCAAGTTTG
4	CEDG133	GCATACATAATGTGGTGAGATG	GTCTCGTGCCTTTCACAC
5	CEDG039	CTATCGAAGTAATACATTCAAAGC	GATCTATGCATGGATCTAGCATC
		Chromosome 2	
6	CEDG225	GAGGAAGTGTTGCAGCACC	GTAGACTCTGCAGAGGGATG
7	CEDG065	GGAATTTTGAGAACGGATTTGC	CCACCGACCACGGCCTTC
8	CEDG284	GGTGCTAACGTTGGAAACTGAG	CACTCCATTCTGAGGATCAATCC
		Chromosome 3	
9	CEDG186	GGATGGGAGAGTAAGAAG	GCATGGCATGATGACTTG
10	CEDG295	CAAAGGTTAGATCCAACATCG	GGTTAGTCATCAACAACTCC
		Chromosome 4	
11	CEDG088	TCTTGTCATTTAGCACTTAGCACG	TTGTTGTTTACTAAGAGCCCGTGT
12	CEDG139	CAAACTTCCGATCGAAAGCGCTTG	GTTTCTCCTCAATCTCAAGCTCCG
13	CEDG008	AGGCGAGGTTTCGTTTCAAG	GCCCATATTTTTACGCCCAC
14	CEDG165	GCTCTGTCAGTTCCCACTAC	GGTCCTGAACCCAGATGAAC
15	CEDC055	CAAACACTTTTGTAACTCCC	GCTTCTAACCTTGATCCTTC

Sl. No.	SSR locus name	Forward primer (5'-3')	Reverse primer (3'-5')
16	CEDG127	GGTTAGCATCTGAGCTTCTTCGTC	CTCCTCACTTGGTCTGAAACTC
		Chromosome 5	
17	CEDG115	GGCTCATTGTACCACTGGATAT	ATGCCTCCTTTCAGGTGATTGT
18	CEDG264	GATTCCCTTCCTAGCTATGG	CTGCTGGACATGAAGATTCAG
		Chromosome 6	
19	CEDG191	CAATAAGCAATCTGTGGAGAG	CTGCAGGAAACTTGGAATTGC
20	CEDG015	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC
21	CEDG248	CAGAACACAAAAGGGTTCTCG	GTGGATTCACTCGCTTCC
		Chromosome 7	
22	CEDG111	TGGAAGTTTCCAAGAGGGTTTTC	TCTCACCACCTTTTACCTTCTCA
23	CEDG143	GATGAACTCGTCTCGCTCATCG	CTGGACGCGTCTACTCAGAC
24	CEDG201	CGGGTAGACAAAGAGATACACG	CTAGCAGAAACAGGAGATCCTC
		Chromosome 8	
25	CEDG247	GTAGACACTGATCATCACC	GACCATCATCGATACGATTC
26	CEDG299	CTGTTACGGCACCTGGAAAG	GCAGAGACACACCTTAACCTTG
27	CEDG257	GACTACTCTCAAGACCAAAG	GATGGTTGTAGATAACACTCC
28	CEDG265	GTAAAACAAACACACAGGAC	GCTCTCAACGAGAATGAAC
29	CEDG269	CTGTTACGGCACCTGGAAAG	GCAGAGACACACCTTAACCTTG
30	CEDG286	CGAGCAGAACACTGATCATG	CCTCTTAGAGGTCATTGCTC
		Chromosome 9	
31	CEDG056	TTCCATCTATAGGGGAAGGGAG	GCTATGATGGAAGAGGGCATGG
32	CEDG304	ACCACTTCATAATCCCTGAG	GTTGCATGCTATATTTTGGTTCAC
33	CEDC080	CACGTTGGAGGAAGTGACGC	CATCGCCACCACAGAACCA
34	CEDG166	GGTACAACATTCTTCTATTTG	GGCTTATGAGTTTATCTTATC
35	CEDG024	CATCTTCCTCACCTGCATTC	TTTGGTGAAGATGACAGCCC
		Chromosome 10	
36	CEDG150	GAAGGGAATGAAAATGAAACCC	GTTCAATCCATTCAGTCTCC
37	CEDG198	CAAGGAAGATGGAGAGAATC	CCTTCTAAGAACAGTGACATG
38	CEDG298	CAGTTCCTAGTTGCATGTG	CTTGGGCTGAATGTTACC
		Chromosome 11	
39	CEDG100	CCCATCAAGTAACTACATAACA	ATGTGGGACTGGACAAATAAAA
40	CEDG273	GTTTAGCTTCTTCTGCTG	CCAAACTGTCAATATCTGC
		Unmapped	
41	CEDG013	CGTTCGAGTTTCTTCGATCG	ACCATCCATCCATTCGCATC

Table 4 List of RAPD and ISSR primers surveyed for BSA.

Sl. No.	RAPD primer	Primer sequence (5'-3')
1.	OPB06	TGCTCTGCCC
2.	OPB07	GGTGACGCAG
3.	OPB08	GTCCACACGG
4.	OPB09	TGGGGGACTC
5.	OPB10	CTGCTGGGAC
6.	OPX01	CTGGGCACGA
7.	OPX02	TTCCGCCACC
8.	OPX03	TGGCGCAGTG
9.	OPX04	CCGCTACCGA
Sl. No.	**ISSR primer**	**Primer sequence (5'-3')**
1.	UBC807	AGAGAGAGAGAGAGAGT
2.	UBC808	AGAGAGAGAGAGAGAGC
3.	UBC810	GAGAGAGAGAGAGAGAT
4.	UBC811	GAGAGAGAGAGAGAGAC
5.	UBC814	CTCTCTCTCTCTCTCTA
6.	UBC816	CACACACACACACACAT
7.	UBC820	GTGTGTGTGTGTGTGTC
8.	UBC824	TCTCTCTCTCTCTCTCG
9.	UBC826	ACACACACACACACACC
10.	UBC287	ACACACACACACACACCG

Results and discussion

Bruchid reaction and DNA polymorphism between parents, VRM (Gg) 1 and TNAU Red

Inter-specific hybridization is generally made to transfer genes of specific agronomic traits (eg. biotic and abiotic stress resistance) from wild species to the cultivated ones. Interspecific hybridization between mung bean [VRM (Gg) 1, bruchid susceptible] and rice bean (TNAU Red, bruchid resistant) had been attempted [26] with the aim of transferring desirable traits (MYMV and bruchid resistance) from wild relatives into cultivated mung bean and were successful in obtaining F1 plants. These F1 individuals had been further advanced to F9 generation through single seed descent method [31] and the resulting seeds of recombinant inbred lines (187 RILs) were used in this study for brcuhid resistance evaluation. TNAU Red seeds displayed complete resistance (0 % damage) while VRM (Gg) 1 seeds were completely damaged (100 % damage) by *C. maculatus*. Based on the seed damage score, the evaluated 187 RILs were categorized as 10 highly resistant (HRP Value), 12 resistant (R), 55 moderately resistant (MR), 89 susceptible (S) and as 21 highly susceptible (HS) lines to bruchid attack (**Figure 1**; **Tables 1** and **2**). A recombinant inbred (RI) line is produced by repeatedly self - pollinating individual progenies of an F2 population [32]. Thus, theoretically, 99 % of the loci of plants in F8 generation (RIL) are homozygous and it is expected to show the Mendelian inheritance pattern of 1(resistant): 1(susceptible) ratio. However, results of the present bruchid bioassay conducted on the RILs were not consistent phenotypically and showed distorted segregation ratio. The abnormal segregations in inter-specific hybridizations might be due to the product of linkage between markers and factor(s) that operate in the pre and post-zygotic phases. It had also suggested that if a gene that causes segregation distortion is segregating in a population, then markers close to it would tend to exhibit distorted ratios as well [33].

Genetic polymorphism surveyed using a total of 56 SSR primers between parents, VRM (Gg) 1 and TNAU Red, showed 41 polymorphic primers, which were further used for BSA.

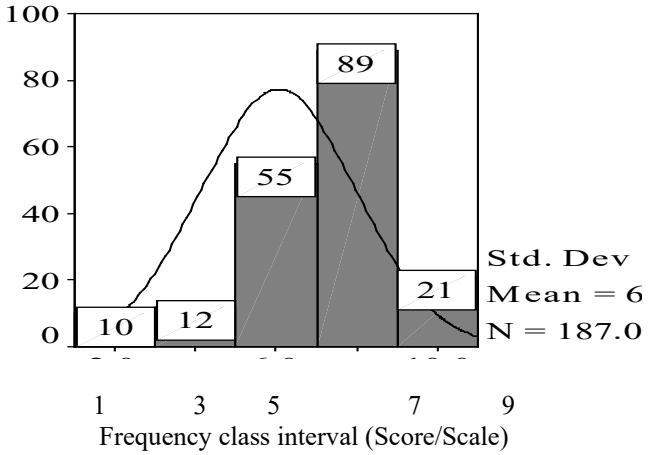


Figure 1 Frequency distribution of 187 RILs based on seed damage per cent.

Table 1 Scoring system for bruchid resistance based on seed damage per cent (Sun *et al*. [29]).

Seed damage %	Level	Score/Scale
0 - 10 %	Highly resistant	1
10.1 - 35 %	Resistant	3
35.1 - 65 %	Moderately resistant or MS	5
65.1 - 90 %	Susceptible	7
> 90 %	Highly susceptible	9

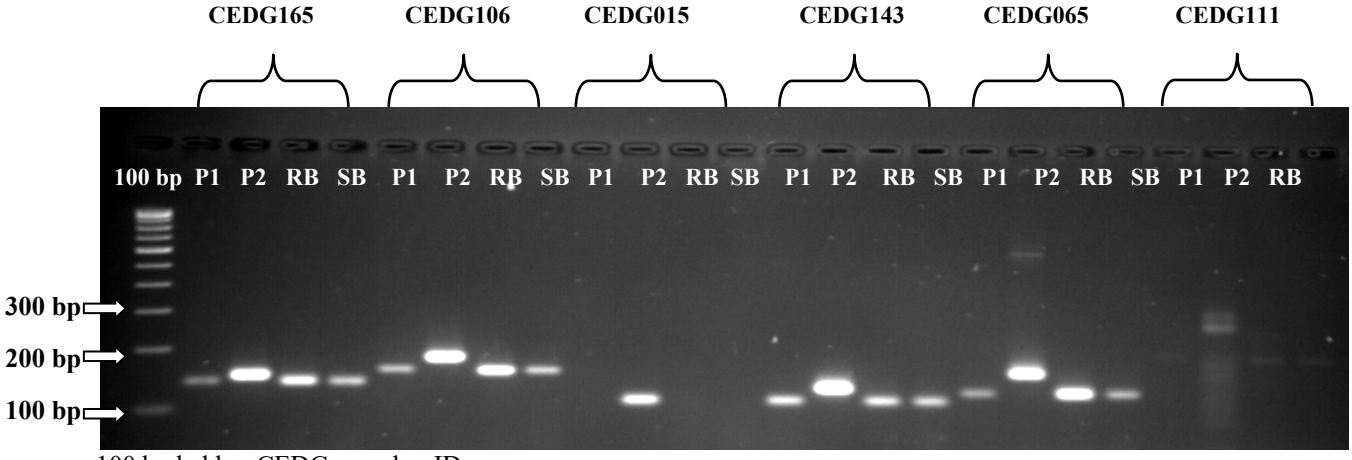
Table 2 Categorization of 187 RILs based on seed damage per cent given by Sun *et al*. [29].

Score	Category (Seed damage %)	Level	No. of RILs	RILs
1	0 - 10	HR	10	**19,** 30, **53**, 74, 83, 120, 149, **158, 165, 169**
3	10.1 - 35	R	12	29, 42, 58, 90, 96, 125, 130, 146, 152, 172, 173, 184,
5	35.1 - 65	MR	55	3, 5, 7, 9, 12, 13, 15, 16, 17, 20, 22, 24, 25, 31, 32, 39, 40, 41, 56, 62, 70, 76, 77, 78, 81, 82, 84, 92, 97, 104, 105, 110, 117, 122, 128, 129, 131, 132, 133, 135, 138, 139, 142, 143, 147, 150, 151, 155, 159, 171, 177, 178, 180, 181, 182
7	65.1 - 90	S	89	1, 4, 6, 8, 11, 18, 21, 23, 26, 27, 28, 33, 34, 35, 36, 37, 38, 43, 44, 45, 46, 47, 48, 49, 50, 51, 54, 55, 57, 59, 61, 63, 65, 67, 68, 69, 71, 79, 80, 85, 86, 89, 91, 94, 95, 98, 99, 100, 101, 102, 103, 106, 107, 108, 109, 111, 113, 114, 115, 118, 119, 124, 126, 127, 134, 136, 137, 140, 141, 144, 145, 148, 153, 154, 156, 157, 160, 161, 162, 164, 167, 168, 170, 174, 176, 179, 185, 186, 187
9	> 90	HS	21	2, 10, 14, 52, **60**, 64, **66**, 72, 73, 75, 87, 88, 93, 112, **116, 121, 123**, 163, 166, 175, 183

Bold RILs - 5 RILs each from resistant (19, 53, 158, 165 and 169) and susceptible (60, 66, 116, 121 and 123) category.

Bulked segregant analysis

Genetic markers represent only the genetic differences between individual organisms or species and they do not represent the target genes themselves [34]. It is reported that markers located near the genes (i.e. closely linked) can be referred to as gene 'tags' and such markers themselves do not affect the phenotype of the trait of interest because they are located only near or 'linked' to genes controlling the trait [35]. In this study, all the 41 azuki bean SSR markers exhibiting polymorphism between VRM (Gg) 1 and TNAU Red were surveyed among resistant and susceptible bulked groups consisting of 5 RILs each. Resistant and highly resistant lines consisted of RIL 53, 83, 149, 158 and 165, whereas RIL 60, 66, 116, 121 and 123 (**Table 2**) were selected as susceptible and highly susceptible lines. The selection of resistant and susceptible RILs also took into account of insect mean developmental period (MDP) in addition to seed damage (SD), which indicates that resistant host seeds delay the normal developmental period of the bruchid insect due to the presence of some anti-nutritive factors, which proved harmful to the pest. The polymorphic SSR primer pairs did not produce the same pattern of polymorphism between susceptible bulk and resistant bulk as seen in susceptible parent and resistant parent. Also, none of the 41 polymorphic SSR markers fit the expected segregation ratio of 1 (resistant): 1 (susceptible). The frequency of VRM (Gg) 1 allele was more for most of the markers and skewed towards its allele. The segregation pattern of some polymorphic SSR markers (CEDG165, CEDG106, CEDG015, CEDG143, CEDG065 and CEDG111) between parental DNA along with 2 extreme DNA bulks is shown (**Figure 2**).



100 bp ladder; CEDG - marker ID
P1 - bruchid susceptible parent, VRM(Gg)1 SB - susceptible bulk (5 lines)
P2 - bruchid resistant parent, TNAU Red RB - resistant bulk (5 lines)

Figure 2 Segregation pattern of azuki bean SSR markers between parental and extreme DNA bulks showing skewness towards susceptible parent.

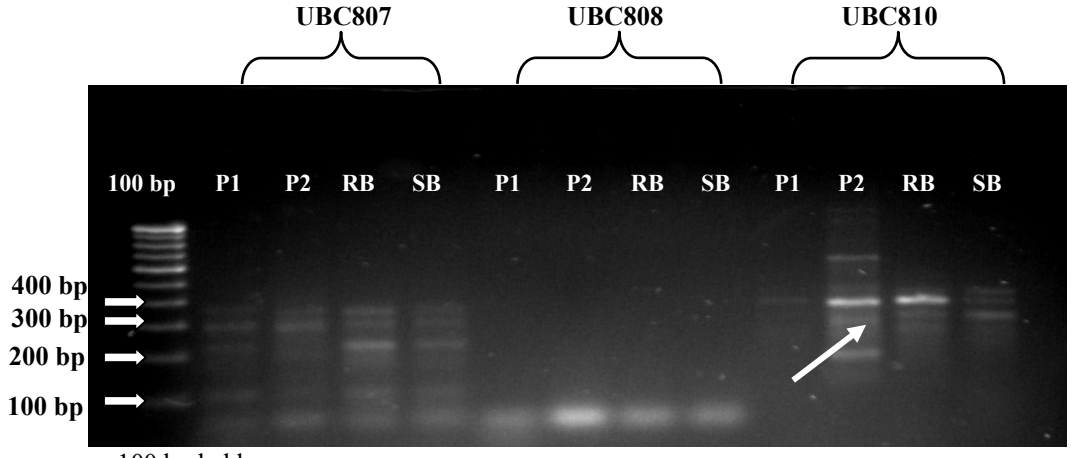
Segregation distortion has been a common observation in studies of mung bean, particularly in crosses between wild and cultivated forms [11]. Several studies conducted with segregating biparental populations reported genomic regions containing loci of minor and major quantitative traits (QTLs) for bruchid resistance. Deviation of markers from the expected segregation ratio (1:1) is a common occurrence that has been observed in inter-specific mapping populations [35]. In previous genetic mapping studies of *Vigna* species such as mung bean, cowpea and azuki bean with distortion levels ranging from 12 to 30.8 % marker segregation distortion was reported [36]. Abnormal segregations in inter-specific hybridizations are the product of linkage between markers and factor(s) that operate in the pre- and post-zygotic phases [35]. They further suggested that if a gene that causes segregation distortion is segregating in a population, then markers close to it would tend to exhibit distorted ratios as well. Similar phenomenon might have taken place in the segregating RIL populations of VRM (Gg) 1 and TNAU Red analysed in the present study.

Segregation of RAPD and ISSR markers

The BSA reduce the burden of surveying all the individuals with polymorphic markers by narrowing the number of markers based on the initial survey of DNA from the parents and the individual bulks of opposite phenotypes [24]. In BSA, DNA from 2 pools (or) bulks from a segregating population

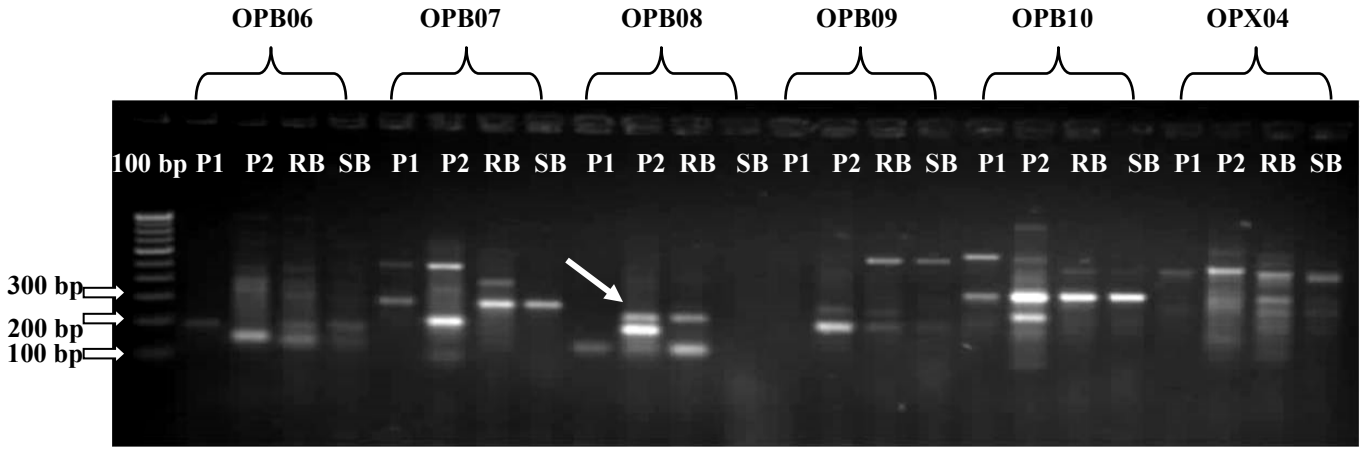
originating from a single cross contrasting for a trait of interest (e.g. insect or disease resistance) are analyzed to identify markers that distinguish them. In this study, BSA was performed with the assumption that within each DNA bulk, the individuals are identical for the bruchid resistant gene (trait of interest in this study) but are different for all other genes. Since the bulks are supposed to show contrast for alleles contributing positive and negative effects, any marker polymorphism between the 2 bulks indicates the linkage between the marker and trait of interest. There are limited or no reports on identification of molecular markers linked with bruchid resistance using bulk segregant analysis in different pulse crops. For instance, 4 closely linked RAPD markers (linked to Br gene) were identified in TC1966 through BSA [37], which were cloned and transformed into sequence characterized amplified region (SCAR) and cleaved amplified polymorphism (CAP) markers. 2 RAPD markers (OPC-06 and STSbr2) linked with another locus (Br2) associated with bruchid resistance were identified in mung bean from 63 RAPD markers and 113 sets of SSR primers using this method [29].

Bulking of DNA from segregants of opposite phenotypes or phenotypic extremes (resistance and susceptible) and analysing them along with parental DNA to identify linked markers was also adopted [38]. In the present experiment, since SSR genotyping of resistant and susceptible bulks did not give the expected results i.e. 1 (resistant): 1 (susceptible) segregation ratio, BSA was conducted using RAPD and ISSR primers. ISSR markers are highly polymorphic and are used in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology [39]. Inter Simple Sequence Repeats-Polymerase Chain Reaction I (SSR-PCR) is a technique which overcomes the problem of low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific SSR primers [40]. Out of 19 (10 ISSR and 9 RAPD) primers surveyed, 3 primers were found to exhibit polymorphism between the resistant and susceptible category. OPB08, OPX04 (RAPD) produced polymorphic band at 200 - 300 bp (**Figure 4**) and UBC810 (ISSR) at 300 - 400 bp (**Figure 3**) base pair lengths in resistant parent as well as in resistant bulk but not in the susceptible parent, susceptible bulk. The amplification of resistant parent allele in resistant bulk may indicate the possession of some rice bean resistant allele in VRM (Gg) 1 X TNAU Red inter-specific populations. Using the same VRM (Gg) 1 X TNAU Red populations (F2 and F3), V. *umbellata* specific SCAR marker (linked with MYMV resistance) was developed from amplified RAPD bands [14] but the *in silico* translated nucleotide sequence of developed SCAR marker showed considerable homology with hypothetical protein of *Escherichia coli*, which was undesirable with regard to the present trait of interest, i.e. bruchid resistant factor. Nonetheless, they proposed that the development of species-specific SCAR markers could pave the way for unambiguous identification of *Vigna* species.



100 bp ladder
P1 - susceptible parent, VRM(Gg)1 SB - susceptible bulk
P2 - resistant parent, TNAU Red RB - resistant bulk

Figure 3 Polymorphic ISSR marker (UBC810) between parental and extreme DNA bulks.



100 bp ladder
P1 - susceptible parent, VRM(Gg)1 SB - susceptible bulk
P2 - resistant parent, TNAU Red RB - resistant bulk

Figure 4 Segregation pattern of RAPD markers between parental and two extreme DNA bulks with two polymorphic markers (OPB08 and OPX04).

In this study, co-segregation analysis was not performed in the remaining resistant and susceptible individuals and species-specific SCAR marker development was not recommended due to the aforesaid complications. As an alternative marker system, the single nucleotide polymorphic markers (SNPs) was used for QTL mapping and candidate gene identification in the same cross *Vigna radiata* X *Vigna umbellata*, which revealed ideal genomic regions associated with bruchid resistance [41]. The BSA based on next generation sequencing (NGS) approaches have been reported to be effective in rapidly mapping genes in different plant species and also help in accelerating QTL mapping [42]. BSA combined with NGS technologies could overcome limits on the availability of DNA markers and thus avoid complete genotyping [25]. It has been reported that this strategy has been successfully used for rapid gene and QTL identification in crops like wheat [43], rice [44], sunflower [45], watermelon [46] and soybean [47]. Furthermore, the combined application of BSA and NGS technologies represents a new way of speeding up the discovery of candidate genes controlling essential agronomic traits in various crops with the availability of sequenced genomes [46].

Conclusions

Advancements in the field of biotechnology and molecular breeding such as marker assisted selection and genetic transformation can be utilized in developing bruchid resistant pulse crops of high economic importance especially mung bean. The associated markers identified in this study can be transformed to Sequence-Characterized Amplified Regions (SCAR) or Cleaved Amplified Polymorphisms (CAPs) to overcome the reproducibility issue associated with RAPDs. These transformed markers will be useful in further characterization of the resistance locus through QTL mapping and candidate gene identification. However, it is recommended to opt for more advanced cost-effective technique such as SNP marker analysis for QTL and gene mapping. Based on the present results, resistant lines have been selected and are being used as donor(s) in bruchid resistance *Vigna* breeding programs at ARS (TNAU), Virinjipuram. This study also confirms the cross-species amplification of SSR markers and the effectiveness of BSA to identify polymorphism associated with bruchid resistance, especially when it is governed by polygenic control. From entomological viewpoint, the present study proved significance in the confirmation of bruchid resistance evaluation.

Acknowledgements

The provision of laboratory equipment and consumables for the molecular studies and supervision by Dr. M Raveendran, Department of Plant Molecular Biology & Biotechnology, Centre for Plant Molecular Biology (CPMB), TNAU, is highly acknowledged. The first author is grateful to Department of Science and Technology (DST-INSPIRE fellowship), New Delhi, India for the financial assistance provided during the entire doctoral programme at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

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