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Genetic linkage mapping of loci conferring resistance to Blue butterfly (*Lampides boeticus* L.) and Plume moth *(Exelastis atomosa* Wals.) on chromosome 2 (CcLG02) in Pigeonpea

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ABSTRACT

Inheritance of host resistance to blue butterfly (PBB_1) and plume moth (PPM_1) in interspecific mapping populations (F_2, F_3 and BC_1) derived from a cross involving *Cajanus cajan* (*cv*. ICP-26) × *Cajanus scarabaeoides* (*acc*. ICPW-94) appeared to be under monogenic control either by a single major gene or a cluster of tightly linked genes. Bulked segregant analysis using 237 [85 simple sequence repeats (SSR), 143 random amplified polymorphic DNA (RAPD) and nine inter simple sequence repeats (ISSR)] parental polymorphic primers led to the identification of 43 markers that distinguished the resistant and susceptible bulks alike to parents, and which were also segregating among F_2 progenies. Linkage analysis of these markers along with interaction phenotype score for both traits generated a linkage group consisting of 11 markers (two SSR, seven RAPD and two ISSR) and two trait loci (PBB_1 and PPM_1). This linkage group distributed over 133.9 cM with an average marker interval of 10.3 cM. The PBB_1 and PPM_1 loci were linked to each other by 11.2 cM (rf 0.110), and were flanked by ISSR marker $UBC872_{2000}$ (15.9 cM), and RAPD marker $OPA09_{910}$ (15.3 cM), respectively. On the basis of sequence homology of linked marker $OPA09_{910}$ these two loci were assigned to chromosome 2 (CcLG02). Composite interval mapping led to the detection of two major quantitative trait loci (*qPBB2.1* and *qPPM2.1*) controlling blue butterfly and plume moth resistance, respectively and the quantitative trait locus peaks coinciding with PBB_1 and PPM_1 loci on the map.

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millsp., Fabaceae) is cultivated as one of the major grain legume crops on 4.92 million hectares of dry and tangential lands in semi-arid tropical and subtropical countries in south Asia, Africa, the Caribbean and Latin America. The global annual production is 3.65 million tons with an average yield of 898 kg/ha (FAOSTAT 2010). The annual yield of this crop has stagnated over the last couple of decades due to various biotic and abiotic stresses (Saxena 2008) together with unadapted agronomic practices and crop management (Varshney et al. 2012). Pigeonpea being a crop of warm humid semi-arid tropics, this crop became a suitable host for various oligo- and poly-phagous pests, mostly those sucking and feeding on inflorescences and pods. A group of such pests [*Helicoverpa armigera* Hubner (pod borer), *Maruca testulalis* Geyer (spotted pod borer), *Lampides boeticus* L. (blue butterfly), *Exelastis atomosa* Wals. (plume moth) and *Melanogromyza obtuse* Malloch. (pod fly)] constitutes a pod borer complex that poses major biotic constraints on achieving sustainable yield in pigeonpea (Grover and Pental 2003; Gnanesh et al. 2011). These pests

cause 30–80% yield losses (Sharma et al. 2010), which accounts for 2.5 million tons of grains per annum, worth more than US$ 300 million per year (Banu, Muthiah, and Ashok 2005). Among these destructive pests, the major loss (70–80%) was due to *H. armigera* (Verulkar, Singh, and Bhattacharya 1997; Banu, Muthiah, and Ashok 2005), and in addition by *L. boeticus* (4–10%) and *E. atomosa* (5–10%) (Sharma et al. 2010). The use of chemicals and pesticides is only partially effective in controlling these pests, and is ecologically harmful and financially not viable. The alternative approach to overcome this problem is the identification and subsequent introgression of host resistance into the promising cultivars, which is an efficient, speedy, reliable and safe means of crop protection. The sources of host resistance to the pod borer and its associated pests including plume moth and blue butterfly were not reported in the primary gene pool (cultivars and local landraces) of pigeonpea so far. However, some of the accessions of *Cajanus scarabaeoides* (L.) Thouars, which belongs to the secondary gene pool (wild, but crossable) possessed host resistance for several pests (Mallikarjuna, Saxena, and Jadhav 2011) including pod borer, caused by *H. armigera*

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(Verulkar, Singh, and Bhattacharya 1997; Aruna et al. 2005; Mishra et al. 2013). Furthermore, these authors reported single dominant genetic control for pod borer resistance in pigeonpea involving inter-specific segregating populations. Recently, a couple of DNA markers linked with pod borer resistance have also been identified in pigeonpea (Mishra et al. 2013).

The use of DNA markers in conventional plant breeding is useful for mapping, monitoring and cloning of agro-economic genes (Kole and Gupta 2004; Varshney, Graner, and Sorrells 2005), and the availability of a tightly linked marker for the desired trait paves the way for introgressive-marker-assisted breeding (Panigrahi et al. 2013). Identification of a DNA marker linked with blue butterfly and plume moth resistance would also allow prompt screening of cultivars, local land races, breeding lines and segregating populations before their use in breeding endeavours aimed at marker-assisted introgression of alleles. There is no report available either on their mode of inheritance or the genetic linkage of DNA markers with blue butterfly and plume moth resistance in pigeonpea.

This study reports on the identification of host resistance against blue butterfly and plume moth in genotypes of *C. scarabaeoides*, and their mode of inheritance using interspecific mapping populations, and identification of loci on a genetic linkage map based on simple sequence repeats (SSR), random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers.

Materials and methods

Screening of a collection of genotypes for host resistance to blue butterfly and plume moth

Seeds of 34 genotypes belonging to genus *Cajanus*, including seven cultivars of *C. cajan* and 27 genotypes of 11 wild species belonging to primary, secondary and tertiary gene pools of pigeonpea, were obtained from ICRISAT, Patancheru, Andhra Pradesh (Table 1). These genotypes were maintained in the experimental garden of the School of Life Sciences, Sambalpur University, Odisha, India (21°29'5" N, 83°52'41" E; altitude 173 m above mean sea level).

In vivo *screening of genotypes*

Ten plants of each genotype were planted in a 2 × 2-m plot in the experimental garden during the third week of October 2010 so that the reproductive phase of the plants coincided with maximum population of pests during February–March 2011. For screening, two to three inflorescences per plant were tagged on the 15th day after flowering, and observations on percentage of buds, flowers and pods infected per inflorescence by

Table 1. Host response of 34 genotypes of *Cajanus* spp. against blue butterfly (*Lampides boeticus*) and plume moth (*Exelastis atomosa) in vivo* (multi-choice field conditions).

Species	Cultivar/Accession Number	Average pod damage (%)		Rating on the basis of field reaction (1–9 scale)	
		Blue butterfly	Plume moth	Blue butterfly	Plume moth
C. cajan	ICP11543	37.03	36.36	9	9
C. cajan	ICP26	36.0	30.30	9	8
C. cajan	ICP28	37.03	30.30	9	8
C. cajan	ICP6971	32.0	36.36	8	9
C. cajan	ICP7439	34.0	35.0	8	9
C. cajan	ICP8858	33.33	30.0	8	8
C. cajan	ICP14722	30.30	34.0	8	8
C. cajanifolius	ICPW31	26.66	24.5	7	6
C. cajanifolius	ICPW30	28.33	24.0	7	6
C. scarabaeoides	ICPW89	0	0	1	1
C. scarabaeoides	ICPW90	0	0	1	1
C. scarabaeoides	ICPW94	0	0	1	1
C. scarabaeoides	ICPW95	0	0	1	1
C. scarabaeoides	ICPW111	0	3.33	1	2
C. scarabaeoides	ICPW115	0	6.66	1	3
C. scarabaeoides	ICPW118	0	0	1	1
C. scarabaeoides	ICPW121	0	0	1	1
C. scarabaeoides	ICPW124	0	3.33	1	2
C. platycarpus	ICPW66	6.66	0	2	1
C. platycarpus	ICPW67	6.66	3.33	2	2
C. platycarpus	ICPW68	3.33	6.66	2	3
C. platycarpus	ICPW69	3.33	3.33	2	2
C. albicans	ICPW17	6.66	14.5	3	4
C. albicans	ICPW13	10.0	20.83	3	6
C. albicans	ICPW24	12.5	18.75	4	5
C. volubilis	ICPW169	13.0	17.5	4	5
C. sericeus	ICPW159	12.5	12.5	4	4
C. sericeus	ICPW160	15.0	15.0	4	4
C. acutifolius	ICPW1	18.46	20.0	5	5
C. lineatus	ICPW44	28.33	24.0	7	6
C. reticulatus	ICPW74	3.33	0	2	1
C. mollis	ICPW54	3.33	3.3	2	2
C. mollis	ICPW58	3.33	2.4	2	2
C. cinereus	ICPW273	0	2.0	1	2

either blue butterfly or plume moth were recorded in the field under multi-choice conditions at the end of the 30th day of tagging. For screening host resistance against blue butterfly and plume moth, the pods and inflorescence present on the tagged stem were screened for exit holes and pin holes, respectively. Accordingly, these genotypes were assigned with interaction phenotype (IP) score on a scale of 1–9 (Sahu et al. 2015), where 1 is completely resistant (no damage) and 9 is highly susceptible (≥ 35% pod damage). The genotypes with IP score 1 to 3 were considered resistant and those with IP score 4 to 9 were considered susceptible to both pests.

In vitro *screening of genotypes*

Larvae and adults of plume moth and blue butterfly were obtained from the colonies maintained in the laboratory for *in vitro* antibiosis assay. The colony of each pest was added separately with their natural population collected from the field to make the larvae populations heterogeneous. The larvae were reared on the chickpea-based artificial diet (Armes, Bond, and Cooter 1992) at 27 ± 1°C, 65 ± 5% relative humidity and 12-hour illumination using cool fluorescent light. The adults were released in 30 × 30 × 30-cm cages. Nappy liners hung inside the cages were provided as a substrate for oviposition and adults were fed with 10% sucrose solution in absorbent cotton. Eggs laid on the nappy liners were sterilized with 1% sodium hypochlorite solution, and transferred into 200-ml plastic cups smeared with a 2-mm-thick layer of artificial diet for rearing in groups of 100 to 150. After 5 days, the larvae were transferred to six-well plates (having 5 ml artificial diet in each well), and reared individually until pupation. The larvae were used for assessing antibiosis to plume moth and blue butterfly by incorporating lyophilized pod powder (10 g/l) into the artificial diet.

Expression of antibiosis was also assessed by rearing respective neonate larvae on artificial diet (Sujana, Sharma, and Rao 2008) augmented with lyophilized pod powder of 34 genotypes with few modifications (Mishra 2013). The blended artificial diet (c. 20 ml) was poured into small glass vials (50 × 100 mm) and fed to these larvae to assess antibiosis response. The rearing cups were kept at 27 ± 2°C, 65 ± 5% relative humidity and 12-hour photoperiod. Each treatment was replicated three times, and there were 10 larvae in each replication. Data on survival of larva and larval weights were recorded on the 10th day after initiating the experiment, as explained above. Similarly, data on weights of pupae were recorded after one day of pupation. Data were recorded on larval and pupal growth periods on the artificial diet. On the basis of larval growth 34 genotypes were assigned an *in vitro* IP score on the 1–9 scale, where 1, ≤ 1.0 mg increment in larval weight; 2, > 1.0 mg and ≤ 5.0 mg increment in larval weight; 3, > 5.1 mg and ≤ 10.0 mg increment in larval weight; 4, > 10.1 mg and ≤ 15.0 mg increment in larval weight; 5, > 15.1 mg and ≤ 20.0 mg increment in larval weight; 6, > 20.1 mg and ≤ 25.0 mg increment in larval weight; 7, > 25.1 mg and ≤ 30.0 mg increment in larval weight; 8, > 30.1 mg and ≤ 35.0 mg increment in larval weight; and 9, ≥ 35.1 mg and above increment in larval weight. Genotypes with score 1 to ≤ 3 were considered resistant, and the genotypes with score > 3 to 9 were considered susceptible.

Development of mapping populations for inheritance studies and linkage analysis

On the basis of plant–pest interaction in field conditions and expression of antibiosis, single plants of *C. cajan cv*. ICP-26 (*in vivo* IP score of 8 and 9 for plume moth and blue butterfly, respectively; *in vitro* IP score of 9 for both blue butterfly and plume moth; susceptible) and *C. scarabaeoides acc*. ICPW-94 (*in vivo* IP score of 1 for both plume moth and blue butterfly; *in vitro* IP score of 3 for both blue butterfly and plume moth; resistance), were self-pollinated and cross-hybridized under a net house during the post rainy season of 2011/12. A single F_1 hybrid plant was self-pollinated to generate the F_2 population (116 progenies), and was crossed with ICP-26 to generate 12 BC_1 plants. F_2 plants were individually self-pollinated to develop 78 F_3 families.

Phenotyping of mapping populations for host resistance

Initially, 116 F_2 progenies and 10 plants from each parent were evaluated for host resistance (field reaction and antibiosis assay) to plume moth and blue butterfly during the pre-rainy season of 2012/13. Twelve plants of the susceptible cultivar *C. cajan cv*. ICP11543 were planted as a control. During the pre-rainy season of 2013/14, the parents, BC_1 plants and progenies of 78 F_3 families were assessed for host resistance to both plume moth and blue butterfly in field conditions only. In addition, all the pods of individual plants were examined for the presence of both exit holes and pin holes at an interval of every 7 days. The evaluation entries were then scored on an IP scale of 1–9, as explained above.

Isolation and purification of genomic DNA

Genomic DNA was isolated from young leaves of parents and F_2 progenies using modified cetyl-trimethyl-ammonium bromide (CTAB) protocol (Sivaramakrishnan et al. 1997), and were equilibrated to a final concentration of 10 ng/μl using $T_{10}E_1$ [10 mM Tris–HCl and 1 mM ethylene diaminetetraacetic acid (EDTA)] buffer. Equal amounts of DNA from ten highly resistant (IP score 1) and ten highly susceptible (IP score of 7 to 9) F_2 progenies for plume moth and blue butterfly were pooled discretely to constitute the resistant bulk and susceptible bulk for both pests, respectively for bulked segregant analysis (Michelemore, Paran, and Kesseli 1991).

RAPD and ISSR marker analysis of the parents and mapping populations

For RAPD and ISSR analysis, polymerase chain reaction (PCR) amplification of 25 ng of genomic DNA was carried out separately using each of 150 decamer oligonucleotide primers (Operon Technologies, Alameda, CA, USA) and 12 synthesized ISSR primers from the set 100/9 (University of British Columbia, Vancouver, Canada), respectively. Each amplification reaction (25 μl) contained the 25-ng template DNA, 2.5 μl of 10× assay buffer [100 mM Tris–HCl pH 8.3; 0.5 M KCl; 0.1% (weight/volume; w/v) gelatine], 1.5 mM MgCl$_2$, 200 μM of each dNTP, 0.25 μM primer, 1.0 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd., India). Amplification conditions for RAPD analysis include an initial denaturation step of 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min, and an extension at 72°C for 2 min; then a final extension was carried out at 72°C for 5 min (Williams et al. 1990). For ISSR analysis, the following PCR conditions were used – an initial denaturation step of 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, a primer annealing at 40–60°C for 45 sec, and an extension at 72°C for 2 min; then a final extension was carried out at 72°C for 5 min (Mishra et al. 2013). Both RAPD and ISSR amplification was carried out in a thermal cycler (Master Cycler Pro; Eppendorf, Hamburg, Germany), and PCR products were separated in 1.4% (w/v) agarose gel containing 0.5 μg/ml ⁻ethidium bromide in TAE buffer (40 mM Tris–acetate, pH 8.0; 2 mM EDTA) at a constant 50 V. A gel loading buffer [20% (w/v) sucrose, 0.1 M EDTA, 1.0% (w/v) sodium dodecyl sulphate, 0.25% (w/v) bromo-phenol blue, 0.25% (w/v) xylene cyanol] was used as a tracking dye. Amplified DNA fragments were visualized on the gel documentation system (Fire Reader Gel Doc System, Uvitec, Cambridge, UK) and photographed. The sizes of the amplified products were determined using a 250-bp step-up ladder (Bangalore Genei Pvt. Ltd., India) as standard, and TL-120 software (Non-linear Dynamics, Total Lab Ltd., Newcastle Upon Tyne, UK). Each marker locus was named by assigning the primer code followed by the size of the fragment in subscript.

SSR marker analysis of the parents and mapping populations

One hundred pairs of synthesized SSR primers (Dutta et al. 2011) were tested to identify polymorphism among ICP-26 and ICPW-94 for further genetic analysis. For SSR analysis, PCR amplifications were carried out in 15-μl volume containing 1.5 μl of 10× assay buffer [160 mM (NH$_4$)$_2$SO$_4$, 500 mM Tris–HCl, pH 9.2; 17.5 mM MgCl$_2$; 0.1% Triton X-100], 133 μM of dNTPs, 10 pmol each of forward and reverse primers, 50 ng of genomic DNA and 0.75 U of *Taq* DNA polymerase (Vivantis

Technologies, Selangor, Malaysia). The PCR cycling profile was initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 7 min. The PCR were performed using gradient PCR (Master Cycler Pro; Eppendorf, Hamburg, Germany). The amplified products were separated in 4% metaphor agarose gels (Lonza, Rockland, ME, USA) containing 0.1 μg/ml ethidium bromide in 1× TBE (40 mM Tris–borate, pH 8.0; 2 mM EDTA) buffer at 120 V for 2 h. A gel loading buffer [20% (w/v) sucrose; 0.1 M EDTA, 1.0% (w/v) sodium dodecyl sulphate; 0.25% (w/v) bromo-phenol blue; 0.25% (w/v) xylene cyanol] was used as a tracking dye. Amplified DNA fragments were visualized and molecular sizes of the amplified products were determined using a 100-bp step-up ladder (Bangalore Genei Pvt. Ltd.) as standard. Each marker locus was named by assigning the primer code (HASSR) followed by the number designated (Dutta et al. 2011).

Segregation and genetic linkage analysis using SSR, RAPD and ISSR markers

SSR (12), RAPD (26) and two ISSR primers distinguishing the parents (ICP-26 and ICPW-94) as well as the bulked samples (resistant bulk and susceptible bulk), were used for single plant analysis of F$_2$ progenies. For the dominant markers, F$_2$ individuals with ICP-26-specific amplified fragment were genotyped as 'A', and individuals without amplified fragments were genotyped as 'B'. In contrast, F$_2$ individuals with ICPW-94-specific amplified fragment were scored as 'B' and vice versa. For co-dominant markers, the F$_2$ individuals with ICP-26-specific amplified fragment were genotyped as 'A', individuals with ICPW-94-specific amplified fragments were genotyped as 'B', and the genotype with both ICP-26- and ICPW-94-specific amplified fragments were genotyped as 'H'. For phenotyping against plume moth and blue butterfly infestation, the F$_2$ progenies were denoted as either ICP-26 type (A) or ICPW-94 type (B). For all cases the missing data were denoted as '–' for genetic linkage analysis.

Linkage relationships among the segregating loci were established using the computer package MAPMAKER/EXP.3.0 (Lincoln, Dally, and Lander 1992). The linkage group was generated using all possible two-point recombination estimates with minimum LOD score of 4.0, and a recombination frequency of 0.30. The most probable marker order was determined using three-point tests followed by the 'order' command. The 'ripple' command was used to verify the marker order. Map distances were expressed in centiMorgan (cM) using the Kosambi map function (Kosambi 1943).

Quantitative trait locus analysis

Genotyping and phenotyping data against blue butterfly and plume moth resistance traits were analysed for mapping quantitative trait loci (QTLs) by using composite

interval mapping (CIM; Zeng 1994) in the Windows QTL Cartographer, version 2.5 (Wang, Basten, and Zeng 2012). CIM analysis was performed using the standard model, scanning the genetic map and estimating the likelihood of a QTL and its corresponding effects at every 1 cM, while using significant marker cofactors to adjust the phenotypic effects associated with other positions in the genetic map. The number of marker cofactors for the background control was set by forward–backward stepwise regression. A window size of 5 cM was used, and therefore cofactors within 2.5 cM on either side of the QTL test site were not included in the QTL model. QTLs were determined to be significant if the corresponding likelihood ratio score was > 11.5 (equal to a LOD score of 2.5). The percent of phenotypic variance explained by a QTL was estimated at the highest probability peaks.

Cloning of linked DNA markers and sequence analysis

Flanking RAPD marker $OPA09_{910}$ was excised from gels using a Genei Pure™ gel extraction kit (Bangalore Genei, Merck Bioscience, India) following the manufacturer's instructions, and the eluted product was used for cloning and sequencing reactions. The purified DNA fragment was ligated into pTZ57R/T vector system and ligation product was transformed into *Escherichia coli* strain JM109 competent cells (Fermentas, Burlington, ON, Canada) using an InsTAclone™ PCR Cloning Kit (ThermoScientific, Waltham, MA, USA) and the manufacturer's instructions. Cells were plated on pre-warmed Luria-Bertani (LB) agar medium containing ampicillin (50 μg/ml), 40 μl of X-gal (20 mg/ml) and 40 μl of iso-propyl β-D-1-thiogalactopyranoside (IPTG) (100 mM) followed by overnight incubation at 37°C. The resulting white colonies on X-gal-IPTG-LB plates containing 50 μg/ml ampicillin were selected, being the putative recombinant clones.

The putative recombinant clones were grown in LB broth containing 50 μg/ml of ampicillin, and plasmids were isolated using a PureSol™ plasmid isolation kit (Bangalore Genei, Merck Bioscience). The plasmids were screened for their insert size by amplification using M13/pUC sequencing primers (S0100 and S0101) following the instructions depicted in the manual. The amplified products were electrophoresed in 1.4% agarose gel and visualized as explained earlier. The cloned fragments was sequenced using a dideoxy chain termination method using M13/pUC-S0100 and M13/pUC reverse S0101 primers at the DNA sequencing Facility (Eurofins Genomics India Pvt. Ltd., Bangalore, India) using a cycle sequencing method in ABI prism 3730 (Applied Biosystem, Foster City, CA, USA) on a hiring basis. The obtained sequence was submitted as query for Basic Local Alignment Search Tool (BLAST) analysis for homology with the deposited sequence of pigeonpea genome databases (http://www.ncbi.nlm.nih.gov/nuccore/ 450342093; http://www.ncbi.nlm.nih.gov/nuccore/ AFSP00000000.1).

Results

Screening of a collection of genotypes for host resistance to blue butterfly and plume moth

Screening under field conditions

Among the 34 genotypes screened under field conditions, 15 wild genotypes belonging to four species of *Cajanus* [*C. scarabaeoides*, *C. platycarpus* (Benth.) van der Maesen, *C. reticulatus* (F.Muell.) van der Maesen and *C. cinereus* (F.Muell.) F.Muell.] showed consistent resistance against both pests with limited pod damage (IP score 1 to 3), whereas all seven genotypes of *C. cajan* appeared as conducive hosts for both pests, and so were grouped under 'susceptible' with IP scores of 8 and 9 (Table 1 and Figure 1). The rest of the genotypes of the secondary and tertiary gene pool showed different degrees of resistance and/or susceptibility to these pests with an IP score varying from 3 to 7.

Screening through in vitro antibiosis assay

There were significant differences in survival rate, larval weight and pupal weight of blue butterfly and plume moth grown on an artificial diet augmented with pod powders of different genotypes *inter se.* The survival rate of plume moth and blue butterfly larvae on an artificial diet based on pod powders of seven cultivars of *C. cajan* varied from 83.3 ± 0.57% to 100.0%, and in the case of the 27 genotypes of the 11 wild species, it varied from 19.6 ± 0.48% to 65.6 ± 0.57% and 20.3 ± 0.57% to 53.3 ± 0.57%, respectively. The average weight of larvae and pupae and the growth period of the two different larvae on artificial diet are enumerated in Table 2 and Figure 2. The plume moth larvae took 23–27 days to complete their development when reared on artificial diet supplemented with pod powders of wild genotypes, compared with 19–23 days with cultivars. The pupal period lasted for 8–9 days for most of the genotypes of *C. scarabaeoides* (including ICPW-94) when reared on artificial diets with pod powders similarly to other genotypes (Figure 2d). A similar trend was also observed for the growth of larvae and pupae of blue butterfly (Table 2 and Figure 2). On the basis of survival and growth of larvae the genotypes were assigned with an IP score on a 1 to 9 scale (Table 2).

Inheritance of host resistance to blue butterfly and plume moth

Following *in vivo* and *in vitro* screening, two genotypes *C. cajan cv*. ICP-26 and *C. scarabaeoides acc*. ICPW-94 were chosen to raise the mapping population. All four F_1 plants generated from the cross (*C. cajan cv*. ICP-26 × *C. scarabaeoides acc*. ICPW-94) were resistant to plume moth and blue butterfly with an IP score '1'. On the basis of simple phenotyping as 'resistant' and 'susceptible',
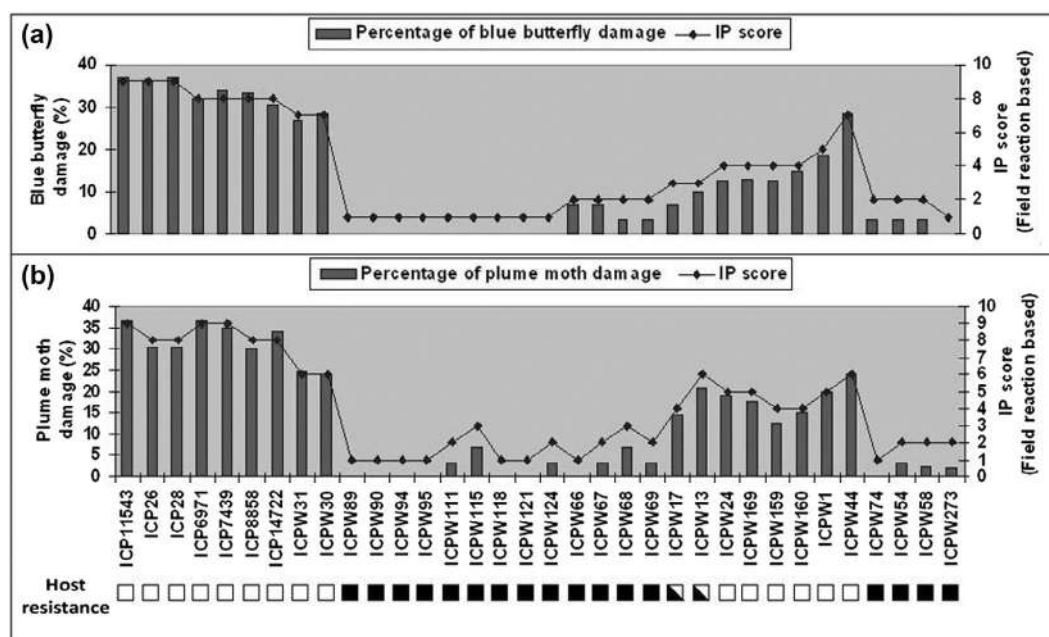
Figure 1. Graphical sketch showing field resistance to blue butterfly (a) and plume moth (b) in terms of percentage of pod damage *vis-a-vis* IP score of 34 genotypes of *Cajanus* spp. (■ resistant to both pests, □, susceptible to both pests and ◣, susceptible to one of the pests).

frequency distribution of 116 F_2 progenies gave a good fit to 3 : 1 for blue butterfly resistance ($\chi^2 = 2.253$; $p = 0.133$) and plume moth resistance ($\chi^2 = 1.655$; $p = 0.198$), indicating single dominant gene control for both blue butterfly and plume moth resistance (Figure 3 and Table 3). In the F_3 generation, the segregation pattern of 78 F_3 families for blue butterfly reaction was a good fit for 1 (20 non-segregating resistant) : 2 (42 segregating) : 1 (16 non-segregating susceptible) ratio ($\chi^2 = 0.872$, $p = 0.832$), which was also evidence for oligo-genic control of blue butterfly resistance. Similar to the segregation seen for blue butterfly, 78 F_3 progenies were also segregated in a 1 (18 non segregating resistant) : 2 (46 segregating) : 1 (14 non-segregating susceptible) ratio ($\chi^2 = 2.923$, $p = 0.403$) for plume moth resistance, showing a monogenic segregation pattern for plume moth resistance. Again a 1 : 1 segregation pattern at BC_1 for both blue butterfly ($\chi^2 = 1.334$, $p = 0.248$) and plume moth ($\chi^2 = 0.332$, $p = 0.564$) resistance confirmed their single dominant gene action. The joint segregation of host resistance to blue butterfly and plume moth also showed significant deviation from the independent assortment ratio ($\chi^2 = 26.037$, $p \le 0.0001$) and were positively associated (Table 3). This was also evidenced by both association analysis (χ^2 of independence values 24.64 with LOD 8.66) and correlation statistics ($r^2 = 0.89$).

Genetic linkage mapping of loci conferring resistance to blue butterfly and plume moth

Among the 262 primers tested, 237 (85 pairs of SSR, 143 RAPD and nine ISSR) primers distinguished the parental lines and were applied for bulked segregant analysis. Out

of them, only 40 parental polymorphic primers, including 12 SSR, 26 RAPD and two ISSR primers, differentiated resistant bulk and susceptible bulk as in the parent (Figure 4 and Table 4), and generated 43 DNA markers (10 SSR, 30 RAPD and three ISSR markers). Of these, 24 markers displayed a simultaneous bulk level polymorphism for both of the pests. Twenty markers including five SSR, 13 RAPD and two ISSR markers were linked to blue butterfly resistance in coupling phase, whereas nine RAPD, one ISSR and five SSR markers were in repulsion phase. Similarly, 16 markers including five SSR, nine RAPD and two ISSR markers were also linked to plume moth resistance in coupling phase, whereas 10 RAPD, one ISSR and five SSR markers were in repulsion phase. Among these markers, 15 (seven SSR, six RAPD and two ISSR) were distorted in their segregation (Table 4). The segregation patterns of three markers, HASSR219, UBC872$_{2000}$ and OPA09$_{910,}$ in a set of 37 F_2 progenies together with host resistance to plume moth and blue butterfly are represented in Figure 5.

Linkage analysis at LOD of 4.0 and recombination frequency 0.3 grouped 11 of the 43 markers identified in the present study in one linkage group along with the loci conferring resistance to blue butterfly (*PBB$_1$*) and plume moth (*PPM$_1$*). This linkage group spanned over 133.9 cM with average marker interval of 10.3 cM. Five markers, including two SSR (HASSR211, HASSR219b), two RAPD (OPB07$_{860}$, OPA07$_{620}$) and one ISSR (UBC872$_{2000}$), showed that segregation distortion remained in this linkage group (Table 5). The *PBB$_1$* and *PPM$_1$* loci were loosely linked (11.2 cM), and flanked by UBC872$_{2000}$ and OPA09$_{910}$ at 15.9 cM and 15.3 cM, respectively (Figure 6). Both the markers UBC872$_{2000}$ and OPA09$_{910}$ were

Table 2. Survival, growth and development of blue butterfly (*Lampides boeticus*) and plume moth (*Exelastis atomosa*) larvae and pupae on artificial diet with lyophilized pod powder of 34 genotypes of *Cajanus* species used in this study.

| Species	Cultivar/ Accession Number	Average larval weight on 10th day (in mg)		Average pupal weight (in mg)		Larval growth period (in days)		Pupal growth period (in days)		Rating on the basis of antibiosis assay on artificial diet (1–9 scale)	
		Blue butterfly	Plume moth	Blue butterfly	Plume moth	Blue butterfly	Plume moth	Blue butterfly	Plume moth	Blue butterfly	Plume moth
C. cajan	ICP11543	118.8 ± 1.0^q	59.4 ± 1.0^m	192.0 ± 1.0^p	96.2 ± 0.8^j	10.6 ± 1.1^a	21.0 ± 1.0^{ab}	10.6 ± 0.5^{ab}	7.0 ± 1.00^{abcd}	9	9
C. cajan	ICP26	75.4 ± 0.4^p	37.7 ± 0.7^l	176.5 ± 0.5^o	88.4 ± 0.4^l	12.0 ± 1.0^{abc}	22.0 ± 1.0^{abc}	10.3 ± 0.5^a	8.0 ± 1.0^{cdef}	9	9
C. cajan	ICP28	75.1 ± 0.1^p	37.5 ± 0.2^{kl}	160.0 ± 1.0^{lm}	79.6 ± 0.6^{gh}	13.0 ± 1.0^{abc}	22.6 ± 0.5^{bcd}	10.3 ± 0.5^a	7.3 ± 0.5^{bcde}	9	9
C. cajan	ICP6971	75.6 ± 1.0^p	37.8 ± 0.8^l	158.3 ± 0.5^l	79.0 ± 2.0^{gh}	12.0 ± 1.0^{abc}	20.3 ± 0.5^a	11.0 ± 1.0^{abc}	7.3 ± 1.1^{bcde}	9	9
C. cajan	ICP7439	73.2 ± 0.2^o	36.3 ± 0.5^l	156.0 ± 0.5^k	78.0 ± 3.0^g	11.3 ± 0.5^{ab}	21.0 ± 1.0^{ab}	11.0 ± 1.0^{abc}	5.6 ± 0.5^a	9	9
C. cajan	ICP8858	71.2 ± 0.2^n	35.5 ± 0.5^k	161.6 ± 0.5^m	81.0 ± 1.0^h	11.3 ± 1.0^{ab}	22.0 ± 2.0^{abc}	10.6 ± 0.5^{ab}	6.6 ± 0.5^{abc}	9	9
C. cajan	ICP14722	69.4 ± 0.4^m	34.7 ± 0.2^k	158.5 ± 0.5^l	79.4 ± 0.4^{gh}	13.0 ± 1.0^{bcd}	23.0 ± 1.0^{bcde}	11.3 ± 0.5^{abc}	6.3 ± 0.5^{ab}	8	8
C. cajanifolius	ICPW31	42.2 ± 1.0^l	21.0 ± 1.0^j	138.5 ± 0.5^g	73.3 ± 4.1^e	12.0 ± 2.0^{abc}	24.0 ± 1.0^{cdefg}	11.6 ± 0.5^{abc}	6.6 ± 0.5^{abc}	6	6
C. cajanifolius	ICPW30	45.8 ± 1.0^k	22.8 ± 1.0^j	142.0 ± 1.0^h	71.5 ± 0.5^{cde}	13.0 ± 2.0^{bcd}	23.0 ± 2.0^{bcde}	10.6 ± 0.5^{ab}	7.0 ± 1.0^{abcd}	6	6
C. scarabaeoides	ICPW89	20.0 ± 0.5^c	10.3 ± 0.3^d	135.0 ± 2.0^{de}	68.0 ± 1.0^{bc}	14.0 ± 2.0^{cde}	25.0 ± 1.0^{defgh}	12.3 ± 0.5^{bcde}	8.0 ± 1.0^{cdef}	3	4
C. scarabaeoides	ICPW90	20.3 ± 0.5^{cd}	12.5 ± 1.0^e	136.4 ± 1.4^{ef}	67.5 ± 0.5^{bc}	15.0 ± 1.0^{def}	23.0 ± 3.0^{bcde}	12.6 ± 0.5^{cde}	8.3 ± 0.5^{def}	4	4
C. scarabaeoides	ICPW94	13.6 ± 1.0^a	5.3 ± 0.3^a	130.0 ± 1.0^a	65.1 ± 0.2^a	16.3 ± 0.5^f	26.6 ± 0.5^h	14.6 ± 0.5^f	9.0 ± 0.0^f	3	3
C. scarabaeoides	ICPW95	14.0 ± 1.0^a	12.6 ± 1.0^e	131.3 ± 0.5^{ab}	66.8 ± 0.9^{abc}	14.0 ± 1.0^{cde}	24.0 ± 1.0^{cdefg}	12.6 ± 0.5^{cde}	8.6 ± 0.5^{def}	3	4
C. scarabaeoides	ICPW111	21.0 ± 0.5^{cd}	10.0 ± 1.0^d	134.0 ± 2.0^{cd}	66.2 ± 0.2^{ab}	15.3 ± 0.5^{ef}	23.3 ± 0.5^{bcdef}	12.6 ± 0.5^{cde}	8.3 ± 0.5^{def}	4	3
C. scarabaeoides	ICPW115	21.5 ± 0.5^d	10.5 ± 1.0^d	134.5 ± 0.5^{cd}	67.6 ± 0.6^{bc}	14.0 ± 1.0^{cde}	24.0 ± 1.0^{cdefg}	12.3 ± 0.5^{bcde}	8.6 ± 0.5^{ef}	4	4
C. scarabaeoides	ICPW118	13.7 ± 1.0^a	15.2 ± 0.2^f	133.0 ± 1.0^{bc}	66.5 ± 0.5^{abc}	15.6 ± 0.5^{ef}	23.0 ± 2.0^{bcde}	13.3 ± 0.5^{def}	7.6 ± 0.5^{bcdef}	3	5
C. scarabaeoides	ICPW121	20.0 ± 0.5^c	5.9 ± 1.0^a	134.0 ± 1.0^{cd}	67.7 ± 0.3^{bc}	13.0 ± 1.0^{bcd}	25.0 ± 1.0^{defgh}	13.6 ± 0.5^{ef}	8.0 ± 1.0^{cdef}	3	3
C. scarabaeoides	ICPW124	13.9 ± 1.0^a	19.1 ± 0.1^h	133.5 ± 0.5^{cd}	67.4 ± 0.4^{bc}	14.0 ± 1.0^{cde}	24.0 ± 3.0^{cdefg}	12.0 ± 1.0^{bcd}	8.6 ± 0.5^{ef}	3	5
C. platycarpus	ICPW66	34.2 ± 0.2^g	7.0 ± 1.0^{ab}	138.2 ± 0.2^{fg}	68.5 ± 0.5^{bc}	15.3 ± 0.5^{ef}	26.0 ± 1.0^{gh}	12.3 ± 1.1^{bcde}	8.3 ± 0.5^{def}	5	3
C. platycarpus	ICPW67	37.8 ± 0.2^h	7.0 ± 1.0^{ab}	138.0 ± 2.0^{fg}	68.4 ± 1.0^{bc}	14.0 ± 1.0^{cde}	25.6 ± 1.0^{fgh}	12.3 ± 1.5^{bcde}	8.0 ± 1.0^{cdef}	5	3
C. platycarpus	ICPW68	38.3 ± 0.3^h	6.8 ± 0.4^{ab}	137.4 ± 1.3^{fg}	68.7 ± 0.7^c	15.3 ± 0.5^{ef}	25.0 ± 1.0^{defgh}	11.3 ± 1.1^{abc}	8.0 ± 1.0^{cdef}	5	3
C. platycarpus	ICPW69	30.4 ± 0.4^f	6.8 ± 1.0^{ab}	137.5 ± 1.0^{fg}	68.2 ± 0.2^{bc}	14.0 ± 1.0^{cde}	25.3 ± 0.5^{efgh}	12.0 ± 2.0^{bcd}	7.6 ± 0.5^{bcdef}	5	3
C. albicans	ICPW17	44.6 ± 0.6^j	22.3 ± 0.3^{ij}	162.0 ± 2.0^n	80.0 ± 1.0^{gh}	13.0 ± 1.0^{bcd}	23.0 ± 1.0^{bcde}	11.3 ± 0.5^{abc}	7.6 ± 0.5^{bcdef}	6	6
C. albicans	ICPW13	48.0 ± 1.0^l	24.0 ± 2.0^l	161.4 ± 0.4^{mn}	80.8 ± 0.8^h	12.0 ± 2.0^{abc}	24.0 ± 1.0^{cdefg}	11.3 ± 0.5^{abc}	8.3 ± 0.5^{def}	6	6
C. albicans	ICPW24	47.3 ± 0.3^l	23.6 ± 1.0^l	161.8 ± 0.2^{mn}	81.2 ± 0.2^h	13.0 ± 2.0^{bcd}	23.3 ± 0.5^{bcdef}	12.0 ± 1.0^{bcd}	6.6 ± 0.5^{abc}	6	6
C. volubilis	ICPW169	25.1 ± 0.1^e	17.0 ± 1.0^g	136.3 ± 0.5^{ef}	67.8 ± 0.8^{bc}	14.0 ± 1.0^{cde}	23.0 ± 1.0^{bcde}	12.3 ± 0.5^{bcde}	7.0 ± 1.0^{abcd}	4	5
C. sericeus	ICPW159	20.5 ± 0.5^{cd}	10.2 ± 1.3^d	152.0 ± 1.0^j	76.0 ± 1.0^f	13.0 ± 1.0^{bcd}	25.0 ± 1.0^{defgh}	12.0 ± 1.0^{bcd}	7.6 ± 0.5^{bcdef}	4	4
C. sericeus	ICPW160	20.3 ± 0.5^{cd}	9.5 ± 1.0^{cd}	151.0 ± 1.0^j	75.5 ± 0.5^f	14.0 ± 1.0^{cde}	22.0 ± 1.0^{abc}	12.3 ± 1.1^{bcde}	6.3 ± 0.5^{ab}	4	3
C. acutifolius	ICPW1	16.3 ± 0.7^b	8.1 ± 1.9^{bc}	142.6 ± 0.6^h	70.8 ± 0.8^d	13.0 ± 1.0^{bcd}	24.0 ± 1.0^{cdefg}	11.0 ± 1.0^{abc}	6.3 ± 0.5^{ab}	3	3
C. lineatus	ICPW44	25.2 ± 0.2^e	10.0 ± 1.0^d	145.8 ± 0.8^l	73.0 ± 1.0^e	14.0 ± 1.0^{cde}	23.3 ± 0.5^{bcdef}	12.6 ± 0.5^{cde}	8.3 ± 0.5^{def}	4	3
C. reticulatus	ICPW74	33.5 ± 0.5^g	7.0 ± 1.0^{ab}	146.0 ± 1.0^l	72.8 ± 0.8^{de}	15.0 ± 1.0^{def}	26.6 ± 0.5^h	12.0 ± 0.0^{bcd}	7.6 ± 1.1^{bcdef}	5	5
C. mollis	ICPW54	33.9 ± 0.9^g	16.7 ± 1.0^{fg}	137.5 ± 0.5^{fg}	68.4 ± 1.4^{bc}	15.3 ± 0.5^{ef}	25.6 ± 0.5^{fgh}	12.3 ± 0.5^{bcde}	7.6 ± 0.5^{bcdef}	5	5
C. mollis	ICPW58	33.7 ± 0.7^g	16.9 ± 0.9^{fg}	137.8 ± 0.8^{fg}	68.6 ± 0.6^c	14.0 ± 1.0^{cde}	25.0 ± 1.0^{defgh}	11.6 ± 0.5^{ef}	6.6 ± 1.1^{abc}	5	5
C. cinereus	ICPW273	14.2 ± 1.0^a	7.1 ± 2.0^a	146.0 ± 1.0^l	72.0 ± 2.0^{de}	16.3 ± 0.5^f	26.6 ± 0.5^h	13.6 ± 0.5^{ef}	8.6 ± 0.5^{ef}	5	5
Control artificial diet		110.0 ± 1.0^q	58.8 ± 0.6^m	160.2 ± 1.0^{lm}	80.0 ± 0.6^{gh}	11.0 ± 1.0^{ab}	21.0 ± 1.0^{ab}	11.0 ± 1.0^{abc}	9.0 ± 0.0^f	3	3
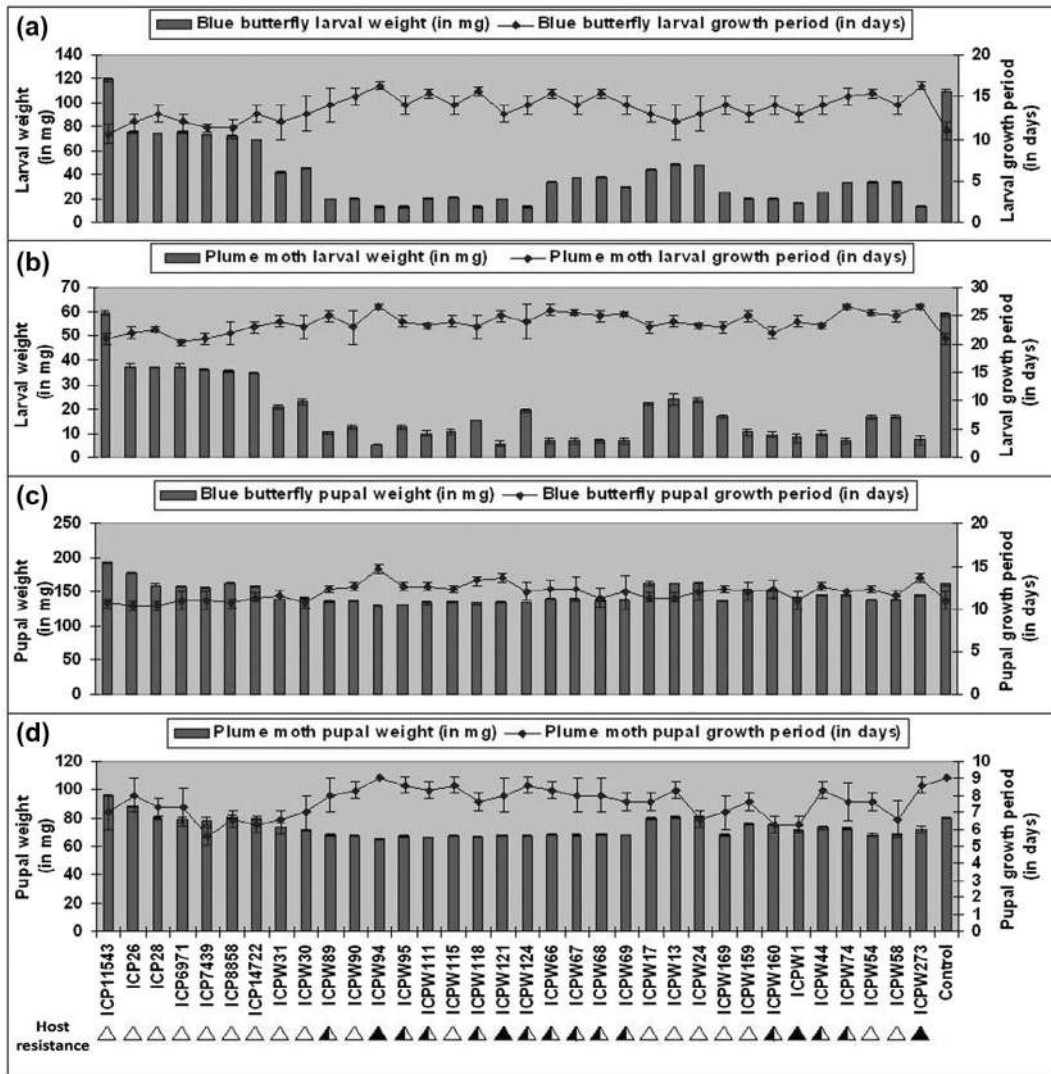
Figure 2. Graphical sketch showing the differences in larval weight (on 10th day), larval growth period, pupal weight and pupal growth period of blue butterfly (a, c) and plume moth (b, d) on AD supplemented with pod powders of 34 genotypes of *Cajanus* spp.
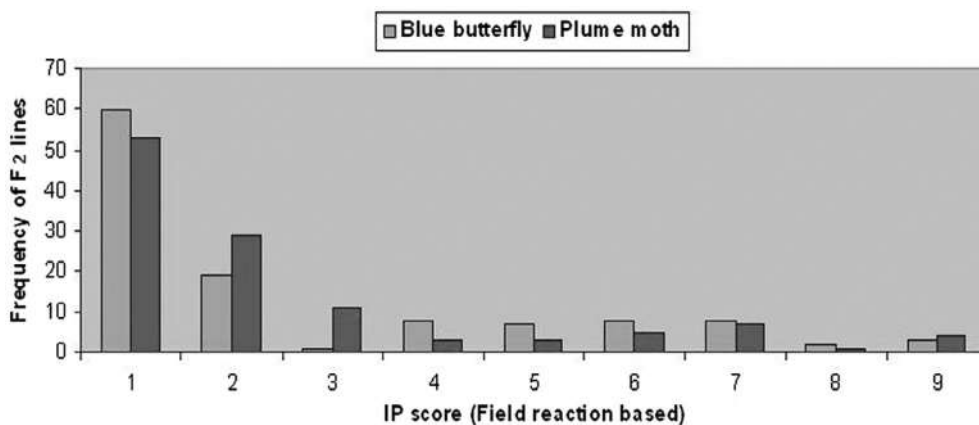
Figure 3. Frequency distribution *F₂* lines derived from cross involving *Cajanus cajan* (ICP-26) and *Cajanus scarabaeoides* (ICPW-94) for IP score with field resistance to blue butterfly and plume moth.

linked to blue butterfly and plume moth resistance in coupling phase with recombination frequency of 0.154 (15.9 cM) and 0.149 (15.3 cM), respectively. However, double recombinants (13.79%) between UBC872$_{2000}$

and OPA09$_{910}$ loci were observed in lower frequency in this F$_2$ population in comparison to single crossovers between *PBB$_1$* and *PPM$_1$* (being linked with recombination frequency 0.110) or with either of the flanked

Table 3. Segregation* and linkage analysis of an interspecific F_2 mapping population, involving *Cajanus cajan* (*cv.* ICP-26) and *Cajanus scarabaeoides* (*acc.* ICPW-94), for resistance to plume moth and blue butterfly.

Field resistance to Plume moth	Field resistance to Blue Butterfly		
	Resistance	Susceptibility	Total
Resistance	74	19	93
Susceptibility	6	17	23
Total	80	36	116

*Segregation for resistance to plume moth ($\chi^2 = 1.655$, $p = 0.198$) and blue butterfly ($\chi^2 = 2.253$, $p = 0.133$) under field conditions tested against 3 : 1; joint segregation ($\chi^2 = 26.037$, $p \leq 0.0001$) tested against 9 : 3 : 3 : 1.
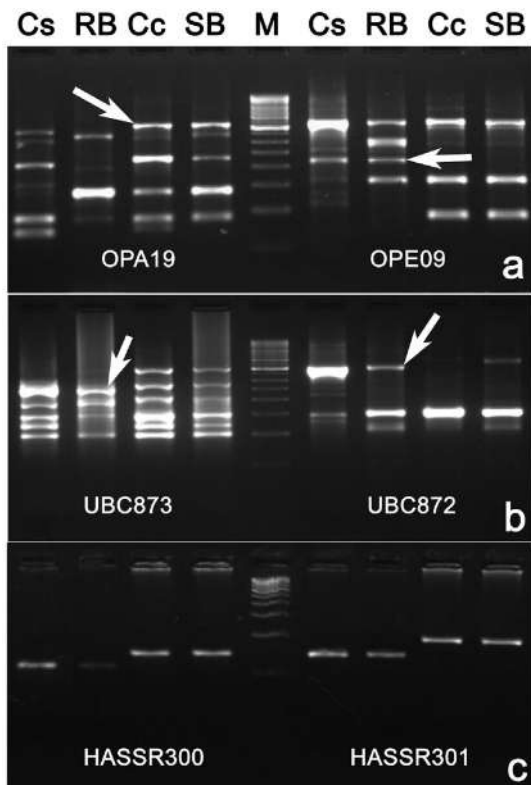

Figure 4. Bulked segregant analysis of F_2 lines using random amplified polymorphic DNA primers – OPA19 and OPE09 (a), ISSR primers – UBC873 and UBC872 (b) and SSR primers HASSR 300 and HASSR 301 (c). Lanes: Cs = resistant parent (*Cajanus scarabaeoides, acc.* ICPW-94), RB = resistant bulk, Cc = susceptible parent (*Cajanus cajan cv.* ICP-26), SB, susceptible bulk; M, 250 bp (a,b) and 100 bp (c) step up ladder, respectively.

markers. For identification of chromosomal location, RAPD marker OPA09$_{910}$ was cloned and sequenced. Sequencing of the marker OPA09$_{910}$ revealed its length to be 942 bp with 41.3% GC content (GenBank accession no. KJ806291.1). BLAST analysis of this sequence with pigeonpea genome sequence showed its position on CcLG02, Scafseq.LG_V5.0fa: 25623599–25622679 bp (Varshney et al. 2012) and contig 01597, AFSP01.fsa.1: 8557–7640 bp (Singh et al. 2012) with more than 99% homology for 2–922 bp sequence of the marker. Hence, the probable chromosome number CcLG02 was assigned to this linkage group.

This framework map was used for composite interval mapping to depict the genetic contribution of the

genomic locations containing resistant loci for blue butterfly or plume moth or both. Scanning the framework linkage map of 13 markers identified one major QTL (*qPBB2.1*) between the confidence interval UBC872$_{2000}$ + 3.3 cM and OPA09$_{910}$ + 15.4 cM at LOD score 41.3 for blue butterfly resistance. This locus explained 74.51% of phenotypic variation with an additive effect –11.801. Similarly, another QTL (*qPPM2.1*) was identified between the confidence interval UBC872$_{2000}$ + 15.6 cM and OPA09$_{910}$ + 0.1 cM at LOD score 45.17 for plume moth resistance. This QTL also explained 69.82% of phenotypic variation with additive effect –12.344. Both the QTL peaks coincided with the major loci identified by a simple phenotyping based linkage analysis (Figure 6).

Discussion

Screening of host resistance for blue butterfly and plume moth, and their gene action

Blue butterfly and plume moth infestation inflated the loss in yield caused by pod borer in pigeonpea. Host plant resistance could be considered the preferred way of managing these pests, and so extensive efforts were made in the last couple of decades to identify the sources of host resistance among the germplasm of pigeonpea. On screening of host resistance among 34 promising genotypes, *C. scarabaeoides acc.* ICPW-94 showed consistent resistance against plume moth and blue butterfly in field conditions *in vivo*, and was also congruous with the antibiosis expression *in vitro*. Moreover, several accessions of *C. scarabaeoides* have already been identified and used as a source of host resistance to pod borer, pod fly and pod wasp in earlier studies (Verulkar, Singh, and Bhattacharya 1997; Sharma, Pampapathy, and Reddy 2003; Aruna et al. 2005; Sujana, Sharma and Rao 2008; Mishra et al. 2013; Rath et al. 2015).

Knowledge of inheritance and number of genes governing the host resistance to blue butterfly and plume moth is essential to formulate efficient breeding strategies in pigeonpea. Hence, the F_1 plants, F_2 interspecific progenies and BC$_1$ lines, derived from a cross involving *C. cajan cv.* ICP-26 (susceptible) and *C. scarabaeoides acc.* ICPW-94 (Resistant), were used in the present attempt. All four F_1 plants were resistant to both plume moth and blue butterfly infestation with an IP score of '1' and their segregation among 116 F_2 progenies gave a good fit to an expected 3 : 1 ratio, which were indicative of a single dominant gene control for both blue butterfly and plume moth resistance. The segregation patterns of 78 F_3 families (1 : 2 : 1) and 12 BC$_1$ plants (1 : 1) were also substantiated for monogenic control of host plant resistance against both blue butterfly and plume moth, as reported for pod borer resistance (Verulkar, Singh, and Bhattacharya 1997; Aruna et al. 2005; Mishra et al. 2013) and fusarium wilt resistance (Kotresh et al. 2006) in pigeonpea. However, the frequency distribution in F_2 progenies with IP score 1 to 9 could not exclude the

Table 4. Information about DNA markers generated by 40 polymorphic primers [RAPD (26) and ISSR (02) and SSR (12)] and their validation through bulked segregant analysis before single plant analysis for linkage studies.

Primer	Primer sequence (5'–3')	Polymorphic fragments originated in ICP-26	Polymorphic fragments originated in ICPW-94	Polymorphic fragments appeared in RB — Plume moth	Polymorphic fragments appeared in RB — Blue butterfly	Polymorphic fragments appeared in SB — Plume moth	Polymorphic fragments appeared in SB — Blue butterfly	Linkage phase
RAPD primers								
OPA-04	5'-AATCGGGCTG-3'	OPA-04$_{2875,640}$	OPA-04$_{1500}$	OPA-04$_{1500}$	OPA-04$_{1500}$	–	–	Coupling
OPA-07	5'-GAAACGGGTG-3'	OPA-07$_{985}$	OPA-07$_{620}$	OPA-07$_{620}$	OPA-07$_{620}$	–	–	Coupling
OPA-08	5'-GTGACGTAGG-3'	OPA-08$_{950}$	–	–	–	–	OPA-08$_{950}$	Repulsion
OPA-09	5'-GGGTAACGCC-3'	OPA-09$_{1415}$	OPA-09$_{910}$	OPA-09$_{910}$	OPA-09$_{910}$	–	–	Coupling
OPA-12	5'-TCGGCGATAG-3'	OPA-12$_{2460}$	–	–	–	OPA-12$_{2460}$	–	Repulsion
OPA-14	5'-TCTGTGCTGG-3'	–	OPA-14$_{800}$	–	OPA-14$_{800}$	–	–	Coupling
OPA-16	5'-AGCCAGCGAA-3'	OPA-16$_{2000}$	OPA-16$_{655}$	–	–	OPA-16$_{2000}$	–	Repulsion
OPA-18	5'-AGGTGACCGT-3'	OPA-18$_{565}$	–	–	–	OPA-18$_{565}$	OPA-18$_{565}$	Repulsion
OPA-19	5'-CAAACGTCG-3'	OPA-19$_{2135,1145}$	OPA-19$_{1950}$	–	–	OPA-19$_{2135}$	OPA-19$_{2135}$	Repulsion
OPA-20	5'-GTTGCGATCC-3'		OPA-20$_{1110}$	–	OPA-20$_{1110}$	–	–	Coupling
OPB-07	5'-GGTGACGCAG-3'	OPB-07$_{620}$	OPB-07$_{860}$	–	–	OPB-07$_{620}$	–	Repulsion
OPB-08	5'-GTCCACACGG-3'	OPB-08$_{2625}$	–	–	–	–	OPB-08$_{2625}$	Repulsion
OPB-12	5'-CCTTGACGCA-3'	OPB-12$_{615}$	OPB-12$_{1070}$	–	OPB-12$_{1070}$*	–	–	Coupling
OPB-15	5'-GGAGGGTGTT-3'	OPB-15$_{435,1050}$	–	–	–	OPB-15$_{1050}$*	OPB-15$_{1050}$*	Repulsion
OPC-02	5'-GTGAGGCGTC-3'	OPC-02$_{2440,510}$	OPC-02$_{1750}$	OPC-02$_{1750}$		OPC-02$_{2440,510}$*	OPC-02$_{2440,510}$*	Coupling / Repulsion
OPC-05	5'-GATGACCGCC-3'	OPC-05$_{960}$	OPC-05$_{2845}$	OPC-05$_{2845}$	OPC-05$_{2845}$	–	–	Coupling
OPC-10	5'-TGTCTGGGTG-3'		OPC-10$_{765}$	–	OPC-10$_{765}$	–	–	Coupling
OPC-14	5'-TGCGTGCTTG-3'	OPC-14$_{760}$	OPC-14$_{1385}$	OPC-14$_{1385}$	–	–	–	Coupling
OPC-17	5'-TTCCCCCCAG-3'		OPC-17$_{1100}$	–	OPC-17$_{1100}$	–	–	Coupling
OPC-20	5'-ACTTCGCCAC-3'	OPC-20$_{910}$	–	–	–	–	OPC-20$_{910}$	Repulsion
OPD-01	5'-ACCGCGAAGG-3'		OPD01$_{2000}$	OPD01$_{2000}$*	OPD01$_{2000}$	–	–	Coupling
OPD-08	5'-GTGTGCCCCA-3'		OPD-08$_{1200}$	OPD-08$_{1200}$*	OPD-08$_{1200}$	–	–	Coupling
OPE-09	5'-CTCTGGAGAC-3'		OPE-09$_{1100}$	OPE-09$_{1100}$*	OPE-09$_{1100}$*	–	–	Coupling
OPM-05	5'-GGGAACGTGT-3'		OPM-05$_{1480}$	–	OPM-05$_{1480}$*	–	–	Coupling
OPM-11	5'-GTCCACTGTG-3'	OPM-11$_{1395,920}$	–	–	–	OPM-11$_{1395}$	OPM-11$_{920}$	Repulsion
OPO-02	5'-ACGTAGGGTC-3'	OPO-02$_{2405}$	OPO-02$_{950}$	–	OPO-02$_{950}$	OPO-02$_{2405}$	–	Coupling/Repulsion
ISSR primers								
UBC-872	5'-(GATA)$_4$-3'	UBC-872$_{775}$	UBC-872$_{2000}$	UBC-872$_{2000}$*	UBC-872$_{2000}$*	UBC-872$_{775}$	UBC-872$_{775}$	Coupling/Repulsion
UBC-873	5'-(GACA)$_4$-3'		UBC-873$_{1270}$	UBC-873$_{1270}$	UBC-873$_{1270}$	–	–	Coupling
SSR primers								
CcM0076	F:CAAAATTAAAGTCAAAGCTTAATCCA, R:TGTTCCTTCAATGGAAAATGA	CcM0076$_{200}$	CcM0076$_{275}$	–	–	–	–	–
CcM0948	F:GCACAGGTCACGTCTGTACC, R:CATTTTCCCACCTTTCCTGA	CcM0948$_{210}$	CcM0948$_{315,185}$	–	–	CcM0948$_{210}$	CcM0948$_{210}$	Repulsion
CcM1596	F:AGATCCAAAATTCACCAACCA, R:CACAAAGATTCAATAATGACACCA	CcM1596$_{275}$	CcM1596$_{250}$	–	–	–	–	–
CcM2236	F:GGAATCGAATGAAAGCCAAA, R:TGCAATGAAAGCACTTACGG	CcM2236$_{300}$	CcM2236$_{345}$	–	–	–	–	–
CcM2763	F:AGCACATCAAACAACAGGTAGA, R:CGCAGCCATTGAAGGAAGTA,	CcM2763$_{220}$	CcM2763$_{260}$	CcM2763$_{260}$	CcM2763$_{260}$	CcM2763$_{220}$	CcM2763$_{220}$	Coupling/Repulsion
CcM2878	F:TTCGTTGCGATCTGAGACTG, R:AATGGAAGAACATGAAGCCG	CcM2878$_{295}$	CcM2878$_{315}$	–	–	–	–	–
HASSR74	F:TCAAAATCCAATATTGTACGG, R:CTGAACAAATGATGATTCAAAC	HASSR74$_{170}$	–	HASSR74$_{170}$	HASSR74$_{170}$	HASSR74$_{170}$	HASSR74$_{170}$	Repulsion
HASSR211	F:CCAAATGTGAAGCGTTTTTA, R:GAAAAGGAAATCTTTTGTTCAC		HASSR211$_{120}$	HASSR211$_{120}$	HASSR211$_{120}$	–	–	Coupling
HASSR219	F:GATGTGGATGTGGAtGTGT, R: ACTTGATTATCCCCTTCCTC		HASSR219b$_{100}$	HASSR219b$_{100}$	HASSR219b$_{100}$	–	–	Coupling
HASSR300	F:CAACCGTTGTCAATGTAAATC, R:GGTCTCTGGTTTACTCAGGTC	HASSR300$_{130}$	HASSR300$_{110}$	HASSR300$_{110}$	HASSR300$_{110}$	HASSR300$_{130}$	HASSR300$_{130}$	Coupling/Repulsion
HASSR301	F:TCGAACTGATCAGAACAAGAC, R:TGAATCTGATGAGAGCACAA	HASSR301$_{190}$	HASSR301$_{135}$	HASSR301$_{135}$	HASSR301$_{135}$	HASSR301$_{190}$	HASSR301$_{190}$	Coupling/Repulsion
HASSR288	F:GACATTCATTTACTCCCGATT, R:GGGTCTCACAAAAACAAATAA	HASSR288$_{125}$	HASSR288$_{100}$	–	–	–	–	–

*Marker loci distorted for segregation against 3 : 1 ratio at p = 0.05
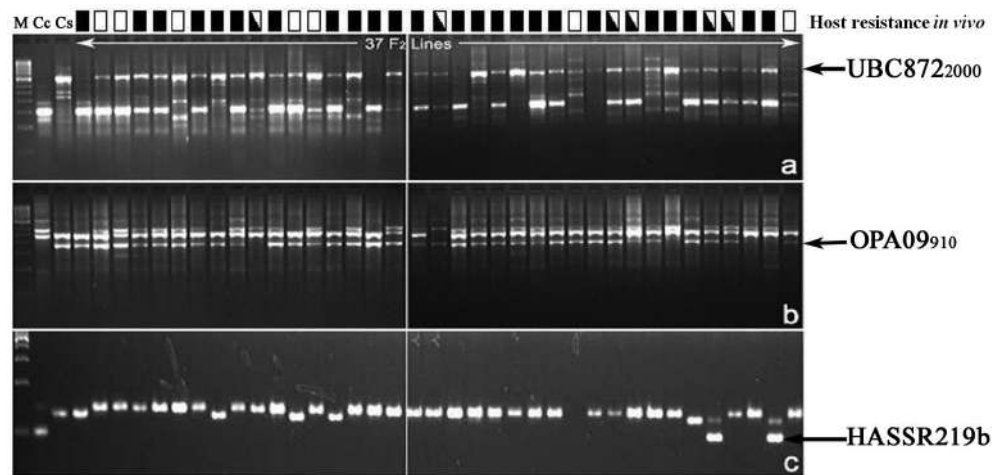ISSR, simple sequence repeat; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat.

Figure 5. Segregation of ISSR marker UBC872$_{2000}$ (a), random amplified polymorphic DNA marker OPA09$_{910}$ (b) and SSR marker HASSR219b (c) in a set of 37 F_2 progenies. Lanes: M, 250 bp step up ladder, Cc = susceptible parent (*Cajanus cajan cv.* ICP-26) and Cs = resistant parent (*Cajanus scarabaeoides*, *acc.* ICPW-94). Reaction to blue butterfly and plume moth (■, resistant to both pests, □, susceptible to both pests and, susceptible to one of the pests) was indicated on the top of the lanes of 37 F_2 lines.

Table 5. Segregation of seven RAPD, two ISSR and two SSR markers (present in the linkage group) in the F_2 interspecific mapping population along with genetic phase of linkage.

| DNA markers | Observed frequency in the F_2 population | | χ^2-value (3:1) | p-value | Linkage phase with respect to PPM_1 and PBB_1 |
	Cajanus cajan (ICP-26) type	*Cajanus scarabaeoides* (ICPW-94) type			
UBC872$_{2000}$	19	94	4.038*	0.044	Coupling
OPA09$_{910}$	20	95	3.550	0.059	Coupling
OPC10$_{765}$	27	86	0.073	0.785	Coupling
OPC02$_{1750}$	23	92	1.533	0.215	Coupling
UBC872$_{775}$	81	32	0.663	0.415	Repulsion
HASSR219b	06	102	21.777*	≤ 0.0001	Coupling
HASSR211	09	93	14.235*	≤ 0.0001	Coupling
OPB07$_{860}$	16	97	7.082*	0.007	Coupling
OPA07$_{620}$	14	88	6.915*	0.008	Coupling
OPD01$_{2000}$	18	84	2.941	0.086	Coupling
OPC17$_{1100}$	26	74	0.053	0.817	Coupling

*Marker loci distorted for segregation against 3:1 ratio at $p = 0.05$
ISSR, inter simple sequence repeat; RAPD, random amplified polymorphic DNA; SSR, simple sequence repeat.

possible involvement of one or more genes for the host resistance conferred by *C. scarabaeoides acc.* ICPW-94. This might be due to simple plant–pest interaction phenotyping such as scoring '1' and '0' for resistant and susceptible in F_2 population, used in this study and this approach will tend to estimate only the major gene(s) governing host resistance. Moreover, the joint segregation studies among host resistance conferred by these two pests deviated significantly from expected 9 : 3 : 3 : 1, which was also an indication towards close linkage between the two loci contributing host resistance and was further confirmed by association analysis.

Molecular mapping of loci conferring blue butterfly and plume moth resistance

The application of bulked segregant analysis with DNA markers including SSR, ISSR and RAPD can facilitate the detection of markers closely linked to the trait of agronomic interest (Michelemore, Paran, and Kesseli 1991).

The segregation of host plant resistance in F_2 progenies and dominant RAPD and ISSR markers in the coupling phase of linkage showed good fit to 3 : 1 ratio baring two RAPD markers (OPB07$_{860}$ and OPA07$_{620}$) and one ISSR marker (UBC872$_{2000}$). The segregation distortion of a few dominant markers could also be attributed to abortion of male or female gametes or selective exclusion of particular gametic genotypes from fertilization, owing to incompatibility, incongruity and preferential zygote selection during interspecific hybridization involving *C. cajan cv.* ICP-26 and *C. scarabaeoides acc.* ICPW-94 (Kreike and Stiekema 1997; Gupta et al. 2006). The codominant SSR marker could distinguish homozygous and heterozygous resistant genotypes among the segregating progenies (Zietkiewicz, Rafalski, and Labuda 1994). In our study, the difference among homozygous and heterozygous resistant plants was depicted by both the SSR markers, except for some F_2 progenies, which points towards the involvement of a common genomic region conferring resistance to both blue butterfly and
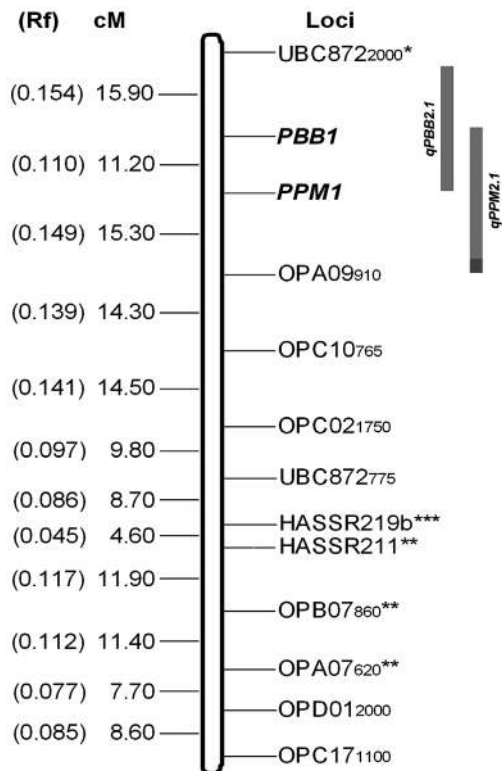
Figure 6. Linkage group indicating the position of DNA markers linked to the locus conferring resistance (*PBB₁*) and plume moth (*PPM₁*) in pigeonpea along with the their respective quantitative trait loci (*qPBB2.1* and *qPPM2.1*); * deviated significantly from 3 : 1 segregation ratio at $p = 0.5$.

plume moth. Moreover, these findings also showed putative genetic linkage of these markers with blue butterfly and plume moth resistance.

Marker-assisted breeding is the foremost application of DNA markers, and one of its primary prerequisites is the establishment of marker to gene linkage through segregating populations (Panigrahi et al. 2013). Identification of DNA marker(s) closely linked to blue butterfly and plume moth resistance should facilitate future breeding efforts aimed at introgression of corresponding alleles in pigeonpea. Only a few reports pertaining to identification of DNA markers linked to qualitative traits, namely fusarium wilt resistance (Kotresh et al. 2006), sterility mosaic disease resistance (Ganapathy et al. 2009), plant type (Dhanasekar, Dhumal, and Reddy 2010) and pod borer resistance (Mishra et al. 2013) were available in pigeonpea. In the present study two loci, PBB_1 and PPM_1, were loosely linked (11.2 cM), and were flanked by two markers UBC872$_{2000}$ and OPA09$_{910}$ with the lowest number of recombinants between themselves. These findings supported the simultaneous use of both UBC872$_{2000}$ and OPA09$_{910}$ in marker-assisted breeding efforts using *C. scarabaeoides acc.* ICPW-94 as donor parent. Besides, the closely linked markers could be converted to sequence characterized amplified region (SCAR) markers for efficient and reliable introgression of host

resistance alleles into the cultivated genetic background without antibiosis expression assay and field resistance screening. As both the markers were linked to PBB_1 and PPM_1 loci at more than 10 cM, only the sequence information of OPA09$_{910}$ was deduced for chromosome assignment. This may further be used to search closely linked markers using genome sequence data of pigeonpea. On the basis of OPA09$_{910}$ sequence homology with whole genome sequence (http://www.ncbi.nlm. nih.gov/nuccore/450342093) of *C. cajan*, both loci (PBB_1 and PPM_1) were assigned to CcLG02 (Varshney et al. 2012). This type of comparative genome analysis was first used for mapping of blast resistance gene in rice (Sharma et al. 2005, 2012) and black rot resistance locus in *Brassica oleracea* var. *botrytis* L. (Saha et al. 2014). Moreover, the scanning of this CcLG02 genome sequence may lead to the development of markers that are closely linked to the host resistance loci, which could help the introgression of alleles with more precision. CIM analyses also identified two major QTLs (*qPBB2.1* and *qPPM2.1*) controlling blue butterfly and plume moth resistance, and these QTLs inherited the 'resistant' allele from the donor parent *C. scarabaeoides acc.* ICPW-94. Both the loci (*qPBB2.1* and *qPPM2.1*) explained 74.51% and 69.82% variation at LOD values of 41.3 and 45.17, respectively. This is not uncommon and has been reported in many crop species including pigeonpea (Gnanesh et al. 2011; Kumawat et al. 2012). Further studies on fine mapping, positional cloning of these loci contributing host resistance against plume moth and blue butterfly, and their epigenome studies elucidate the molecular mechanism of host resistance in pigeonpea.

Conclusion

This study is the first report on the mode of inheritance of host resistance against blue butterfly and plume moth in pigeonpea, and these resistances appeared to be under the control of a major gene, but the frequency distribution pattern vis-à-vis the QTL analysis suggested the involvement of several minor genes. Both the major resistance gene loci (PBB_1 and PPM_1) along with 11 DNA markers constitute a linkage group, and the linkage group spans over 133.9 cM with an average marker interval of 10.3 cM. The PBB_1 and PPM_1 loci were linked to each other by 11.2 cM (rf 0.110), and were flanked by UBC872$_{2000}$ (15.9 cM), and OPA09$_{910}$ (15.3 cM), respectively. On the basis of sequence homology of the linked marker OPA09$_{910}$, these two resistance loci were assigned to chromosome 2 (CcLG02).

Acknowledgements

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